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April 07, 2005

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APPLICATION NUMBER: 60/609,516

FILING DATE: *September 13, 2004*

RELATED PCT APPLICATION NUMBER: *PCT/US05/08428*



Certified by

Under Secretary of Commerce
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02570 U.S. PTO
091304

PTO/SB/16 (04-04)
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PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53 (c).

Express Mail Label No. EV 513561665 US

| INVENTOR(S) | | | | | |
|--|------------------------|---|--|--|--|
| Given Name (first and middle [if any]) | Family Name or Surname | Residence (City and either State or Foreign Country) | | | |
| Walter F. | Lima | San Diego, CA | | | |
| Josh G. | Nichols | Carlsbad, CA | | | |
| Additional inventors are being named on the _____ separately numbered sheets attached hereto | | | | | |
| TITLE OF THE INVENTION (500 characters max) | | | | | |
| COMPOSITIONS AND METHODS FOR OPTIMIZING CLEAVAGE OF RNA BY RNASE H | | | | | |
| Direct all correspondence to: CORRESPONDENCE ADDRESS | | | | | |
| <input checked="" type="checkbox"/> Customer Number | | 34138 | | | |
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| City | | State | | Zip | |
| Country | | Telephone | | Fax | |
| ENCLOSED APPLICATION PARTS (check all that apply) | | | | | |
| <input checked="" type="checkbox"/> Specification Number of Pages | | 120 | | <input type="checkbox"/> CD(s), Number _____ | |
| <input checked="" type="checkbox"/> Drawing(s) Number of Sheets | | 8 | | <input type="checkbox"/> Other (specify) _____ | |
| <input type="checkbox"/> Application Data Sheet. See 37 CFR 1.76 | | | | | |
| METHOD OF PAYMENT OF FILING FEES FOR THIS PROVISIONAL APPLICATION FOR PATENT | | | | | |
| <input type="checkbox"/> Applicant claims small entity status. See 37 CFR 1.27. | | | | FILING FEE Amount (\$) | |
| <input type="checkbox"/> A check or money order is enclosed to cover the filing fees | | | | | |
| <input checked="" type="checkbox"/> The Director is hereby authorized to charge filing fees or credit any overpayment to Deposit Account Number: 50-1275 | | | | 160 | |
| <input checked="" type="checkbox"/> Payment by credit card. Form PTO-2038 is attached. | | | | | |
| The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government. | | | | | |
| <input checked="" type="checkbox"/> No. | | | | | |
| <input type="checkbox"/> Yes, the name of the U.S. Government agency and the Government contract number are: _____. | | | | | |

Respectfully submitted, Michael P. Strah
SIGNATURE
TYPED or PRINTED NAME Michael P. Straher
TELEPHONE 215 665 2000

[Page 1 of 2]

Date September 13, 2004
REGISTRATION NO. 38,325
(if appropriate)
Docket Number: ISIC0048-001

USE ONLY FOR FILING A PROVISIONAL APPLICATION FOR PATENT

This collection of information is required by 37 CFR 1.51. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 8 hours to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.

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PROVISIONAL APPLICATION COVER SHEET
Additional Page

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Docket Number **ISIC0048-001 (CORE0037US.L3)**

| INVENTOR(S)/APPLICANT(S) | | |
|--|-------------------|---|
| Given Name (first and middle (if any)) | Family or Surname | Residence (City and either State or Foreign Country) |
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| Tadensz K. | Wyrzykiewicz | Carlsbad, CA |
| Balkrishen | Bhat | Carlsbad, CA |
| Stanley T. | Crooke | Carlsbad, CA |
| | | |

[Page 2 of 2]

Number _____ of _____

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FEE TRANSMITTAL for FY 2004

Effective 10/01/2003. Patent fees are subject to annual revision.

☐ Applicant claims small entity status. See 37 CFR 1.27

TOTAL AMOUNT OF PAYMENT (\$) 160

Complete if Known

| | |
|----------------------|------------------------------|
| Application Number | not assigned |
| Filing Date | September 13, 2004 |
| First Named Inventor | Walter F. Lima |
| Examiner Name | not assigned |
| Art Unit | not assigned |
| Attorney Docket No. | ISIC0048-001 (CORE0037US.L3) |

METHOD OF PAYMENT (check all that apply)

☐ Check ☐ Credit card ☐ Money ☐ Other ☐ None
Order

☒ Deposit Account:

Deposit Account Number 50-1275

Deposit Account Name Cozen O'Connor

The Director is authorized to: (check all that apply)

☒ Charge fee(s) indicated below ☒ Credit any overpayments
☒ Charge any additional fee(s) during the pendency of this application
☐ Charge fee(s) indicated below, except for the filing fee to the above-identified deposit account.

FEE CALCULATION

1. BASIC FILING FEE

| Large Entity | | Small Entity | | Fee Description | Fee Paid |
|--------------|----------|--------------|----------|------------------------|-----------|
| Fee Code | Fee (\$) | Fee Code | Fee (\$) | | |
| 1001 | 770 | 2001 | 385 | Utility filing fee | |
| 1002 | 340 | 2002 | 170 | Design filing fee | |
| 1003 | 530 | 2003 | 265 | Plant filing fee | |
| 1004 | 770 | 2004 | 385 | Reissue filing fee | |
| 1005 | 160 | 2005 | 80 | Provisional filing fee | 160 |
| SUBTOTAL (1) | | | | | (\$) 160 |

2. EXTRA CLAIM FEES FOR UTILITY AND REISSUE

Total Claims -20 ** = 0 X Fee from below = 0
Independent Claims -3 ** = 0 X Fee from below = 0
Multiple Dependent X Fee from below = 0

| Large Entity | | Small Entity | | Fee Description |
|--------------|----------|--------------|----------|--|
| Fee Code | Fee (\$) | Fee Code | Fee (\$) | |
| 1202 | 18 | 2202 | 9 | Claims in excess of 20 |
| 1201 | 86 | 2201 | 43 | Independent claims in excess of 3 |
| 1203 | 290 | 2203 | 145 | Multiple dependent claim, if not paid |
| 1204 | 86 | 2204 | 43 | ** Reissue independent claims over original patent |
| 1205 | 18 | 2205 | 9 | ** Reissue claims in excess of 20 and over original patent |

SUBTOTAL (2) (\$) 0

**or number previously paid, if greater; For Reissues, see above

FEE CALCULATION (continued)

3. ADDITIONAL FEES


| Large Entity | | Small Entity | | Fee Description | Fee Paid |
|--------------|----------|--------------|----------|--|----------|
| Fee Code | Fee (\$) | Fee Code | Fee (\$) | | |
| 1051 | 130 | 2051 | 65 | Surcharge - late filing fee or oath | |
| 1052 | 50 | 2052 | 25 | Surcharge - late provisional filing fee or cover sheet. | |
| 1053 | 130 | 1053 | 130 | Non-English specification | |
| 1812 | 2,520 | 1812 | 2,520 | For filing a request for reexamination | |
| 1804 | 920* | 1804 | 920* | Requesting publication of SIR prior to Examiner action | |
| 1805 | 1,840* | 1805 | 1,840* | Requesting publication of SIR after Examiner action | |
| 1251 | 110 | 2251 | 55 | Extension for reply within first month | |
| 1252 | 420 | 2252 | 210 | Extension for reply within second month | |
| 1253 | 950 | 2253 | 475 | Extension for reply within third month | |
| 1254 | 1,480 | 2254 | 740 | Extension for reply within fourth month | |
| 1255 | 2,010 | 2255 | 1,005 | Extension for reply within fifth month | |
| 1401 | 330 | 2401 | 165 | Notice of Appeal | |
| 1402 | 330 | 2402 | 165 | Filing a brief in support of an appeal | |
| 1403 | 290 | 2403 | 145 | Request for oral hearing | |
| 1451 | 1,510 | 1451 | 1,510 | Petition to institute a public use proceeding | |
| 1452 | 110 | 2452 | 55 | Petition to revive - unavoidable | |
| 1453 | 1,330 | 2453 | 665 | Petition to revive - unintentional | |
| 1501 | 1,330 | 2501 | 665 | Utility issue fee (or reissue) | |
| 1502 | 480 | 2502 | 240 | Design issue fee | |
| 1503 | 640 | 2503 | 320 | Plant issue fee | |
| 1460 | 130 | 1460 | 130 | Petitions to the Commissioner | |
| 1807 | 50 | 1807 | 50 | Processing fee under 37 CFR 1.17 (q) | |
| 1806 | 180 | 1806 | 180 | Submission of Information Disclosure Stmt | |
| 8021 | 40 | 8021 | 40 | Recording each patent assignment per property (times number of properties) | |
| 1809 | 770 | 2809 | 385 | Filing a submission after final rejection (37 CFR § 1.129(a)) | |
| 1810 | 770 | 2810 | 385 | For each additional invention to be examined (37 CFR § 1.129(b)) | |
| 1801 | 770 | 2801 | 385 | Request for Continued Examination (RCE) | |
| 1802 | 900 | 1802 | 900 | Request for expedited examination of a design application | |

Other fee (specify) _____

*Reduced by Basic Filing Fee Paid

SUBTOTAL (3) (\$) 0

SUBMITTED BY

| | | | | | |
|-------------------|---|-----------------------------------|--------------------|-----------|--------------|
| Name (Print/Type) | Michael P. Straher | Registration No. (Attorney/Agent) | 38,325 | Telephone | 215 665 2000 |
| Signature |  | Date | September 13, 2004 | | |

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COMPOSITIONS AND METHODS FOR OPTIMIZING CLEAVAGE OF RNA BY RNASE H

5

Field of the Invention

The present invention provides compositions and methods for the optimization of cleavage of RNA species by RNase H. In some embodiments, the invention provides oligonucleotides that possess two or more regions of differing conformation, and at least one transitional nucleobase positioned between the regions that is capable of modulating transfer of the helical conformation characteristic of the region bound to the 3'hydroxy thereof, to the region bound to the 5' hydroxyl thereof.

Background of the Invention

15 RNase H hydrolyzes RNA in RNA-DNA hybrids. RNase H activity appears to be ubiquitous in eukaryotes and bacteria. Although RNases H constitute a family of proteins of varying molecular weight, the nucleolytic activity and substrate requirements appear to be similar for the various isotypes. For example, all RNases H studied to date function as endonucleases exhibiting limited sequence specificity and requiring divalent cations (e.g.,
20 Mg^{2+} , Mn^{2+}) to produce cleavage products with 5'-phosphate and 3'-hydroxyl termini.

Recently, two human RNase H genes have been cloned and expressed. RNase H1 is a 286 amino acid protein and is expressed ubiquitously in human cells and tissues. The amino acid sequence of human RNase H1 displays strong homology with RNase H1 from yeast, chicken, *E.coli* and mouse. Human RNase H2 shares strong amino acid sequence
25 homology with RNase H2 from *C. elegans*, yeast and *E. coli*. Although the biological roles for the human enzymes are not fully understood, RNase H2 appears to be involved in *de novo* DNA replication and RNase H1 has been shown in mice to be important for mitochondrial DNA replication.

The structure of human RNase H1 was shown to consist of a 73 amino acid region
30 homologous with the RNA-binding domain of yeast RNase H1 at the amino-terminus of the protein and separated from the catalytic domain by a 62 amino acid spacer region. The catalytic domain is highly conserved with the amino acid sequences of other RNase H1

proteins and contains the key catalytic and substrate binding residues required for activity. Site-directed mutagenesis of human RNase H1 revealed that the spacer region was required for RNase H activity. Although the RNA-binding domain was shown not to be required for RNase H activity, this region was responsible for the enhanced binding
5 affinity of the human enzyme for the heteroduplex substrate as well as the strong positional preference for cleavage exhibited by the enzyme. The RNA-binding domain of human RNase H1 is conserved in other eukaryotic RNases H1 and the highly conserved lysines at positions 59 and 60 of human RNase H1 have been shown to be important for binding to the heteroduplex substrate. The conserved tryptophan at position 43 was
10 responsible for properly positioning the enzyme on the substrate for catalysis.

Human RNase H1 exhibits a strong positional preference for cleavage, i.e., human RNase H1 cleaves the heteroduplex substrate between 7 to 12 nucleotides from the 5'-RNA/3'-DNA terminus. Based on site-directed mutagenesis of both human RNase H1 and the heteroduplex substrate, the RNA-binding domain was shown to be responsible for the
15 observed positional preference for cleavage. The RNA-binding domain of human RNase H1 appeared to bind to the 3'-DNA/5'-RNA pole of the heteroduplex substrate with the catalytic site of the enzyme positioned slightly less than one helical turn from the RNA-binding domain. Substitution of either the terminal 3'-DNA with a single ribonucleotide or 5'-RNA with a 2'-methoxyethoxy deoxyribonucleotide was shown to cause a concomitant
20 3'-shift of the first 5'-cleavage site on the RNA, suggesting that altering duplex geometry interferes with proper positioning of the enzyme on the heteroduplex for cleavage.

Although the interaction between the RNA-binding domain and the heteroduplex substrate has been characterized, the mechanism by which the catalytic domain of RNase H1 recognizes the substrate has not been fully elucidated.

25 Human RNase H1 is a nuclease that cleaves RNA exclusively in an RNA/DNA duplex via a double-strand DNase cleavage mechanism. Neither double-strand RNA (dsRNA) or DNA (dsDNA) duplexes support RNase H1 activity. The observed structural differences between the RNA/DNA heteroduplex and dsRNA and dsDNA duplexes suggest a possible role for the helical geometry and the sugar conformation of the DNA
30 and RNA in the selective cleavage of the heteroduplex substrate by human RNase H1. Specifically, the deoxyribonucleotides within dsDNA form a southern C_{2'}-endo sugar conformation resulting in a B-form helical conformation, whereas ribonucleotides within

dsRNA form a northern C_{3'}-*endo* pucker and an A-form helical geometry. In contrast, the deoxyribonucleotides of the RNA/DNA heteroduplex have been shown to adopt an eastern O_{4'}-*endo* sugar pucker resulting in a helical conformation where the RNA strand adopts A-form geometry and the DNA strand shares both the A- and B-form helical conformations.

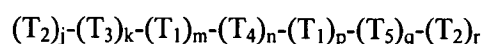
5 The conformational diversity observed for the DNA strand is likely a function of the intrinsic flexibility of the deoxyribonucleotide compared to RNA, and may also be important for human RNase H1 activity. DNA also differs from RNA in that the furanose ring of deoxynucleotide is much more flexible, i.e., exhibit a nearly symmetrical potential energy barrier for both south and north sugar conformations.

10 Consistent with these observations, heteroduplexes containing 2'-ara-fluoro deoxyribonucleotides, which have been shown to exhibit a sugar conformation comparable to DNA when hybridized to RNA, have also been shown to support RNase H1 activity. On the other hand, heteroduplexes consisting of RNA/2'-alkoxy modified deoxyribonucleotides, exhibiting C_{3'}-*endo* sugar pucker and an A-form helical geometry
15 when hybridized to RNA do not support human RNase H1 activity. It has previously been shown that both *E. coli* and human RNases H1 bind A-form duplexes (e.g., RNA/RNA, 2'-methoxyethoxy/RNA and 2'-methoxy/RNA) with comparable affinity to the DNA/RNA heteroduplex substrate but do not cleave the A-form duplexes. In this case, the size and position of the 2'-substituents of RNA and 2'-alkoxy nucleotides suggest possible
20 steric interference with RNase H1 as the 2'-substituents are positioned within the minor groove of the heteroduplex; a region predicted to be the binding site for the enzyme. Alternatively, the sugar conformation and flexibility map play a decisive role in RNase H1 activity.

It can be seen that optimizing the cleavage of RNase H targets would be of great
25 benefit. This invention is directed to this, as well as other, important ends.

Summary of the Invention

In some embodiments, the invention provides compounds of the Formula:



30 wherein

each T₁ is a 2'-deoxyribonucleotide;

each T_2 is a nucleotide having a higher binding affinity for a RNA target as compared to the binding affinity of a 2'-deoxyribonucleotide for said RNA target;

each T_3 , T_4 and T_5 are transitional nucleotides;

j and r independently are 0 to 10, and together the sum of j and r is at least 2;

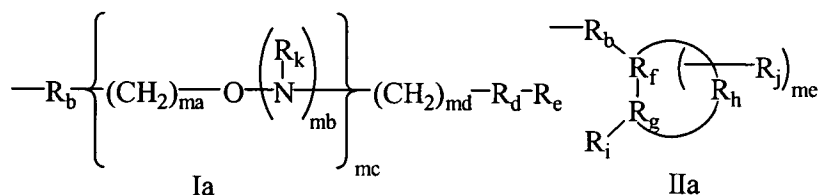
5 m and p independently are 1 to 20, and together the sum of m and p is at least 5;

k , n and q independently are 0 to 3, and together the sum of k , n and q is at least 1.

In some embodiments, T_2 comprises a nucleotide having a northern conformation.

In some such embodiments, T_2 comprises a nucleotide having a 2'-modification. In some further embodiments, the 2'-modification is hydroxyl, -O-alkyl, -O-alkyl-O-alkyl, S-alkyl,

10 S-alkyl-O-alkyl, -F, -O-CH₂CH₂-O-CH₃, -O-CH₃, -O-CH₂-CH=CH₂ or a group having one of formula I_a or II_a:

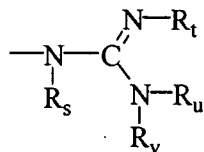


wherein:

15 R_b is O, S or NH;

R_d is a single bond, O, S or C(=O);

R_e is C₁-C₁₀ alkyl, N(R_k)(R_m), N(R_k)(R_n), N=C(R_p)(R_q), N=C(R_p)(R_r) or has formula III_a;



20

III_a

R_p and R_q are each independently hydrogen or C₁-C₁₀ alkyl;

R_r is -R_x-R_y;

each R_s, R_t, R_u and R_v is, independently, hydrogen, C(O)R_w, substituted or unsubstituted C₁-C₁₀ alkyl, substituted or unsubstituted C₂-C₁₀ alkenyl, substituted or

25 unsubstituted C₂-C₁₀ alkynyl, alkylsulfonyl, arylsulfonyl, a chemical functional group or a conjugate group, wherein the substituent groups are selected from hydroxyl, amino,

alkoxy, carboxy, benzyl, phenyl, nitro, thiol, thioalkoxy, halogen, alkyl, aryl, alkenyl and alkynyl;

or optionally, R_u and R_v , together form a phthalimido moiety with the nitrogen atom to which they are attached;

5 each R_w is, independently, substituted or unsubstituted C_1 - C_{10} alkyl, trifluoromethyl, cyanoethoxy, methoxy, ethoxy, t-butoxy, allyloxy, 9-fluorenylmethoxy, 2-(trimethylsilyl)-ethoxy, 2,2,2-trichloroethoxy, benzyloxy, butyryl, iso-butyryl, phenyl or aryl;

R_k is hydrogen, a nitrogen protecting group or $-R_x-R_y$;

10 R_p is hydrogen, a nitrogen protecting group or $-R_x-R_y$;

R_x is a bond or a linking moiety;

R_y is a chemical functional group, a conjugate group or a solid support medium;

each R_m and R_n is, independently, H, a nitrogen protecting group, substituted or unsubstituted C_1 - C_{10} alkyl, substituted or unsubstituted C_2 - C_{10} alkenyl, substituted or
15 unsubstituted C_2 - C_{10} alkynyl, wherein the substituent groups are selected from hydroxyl, amino, alkoxy, carboxy, benzyl, phenyl, nitro, thiol, thioalkoxy, halogen, alkyl, aryl, alkenyl, alkynyl; NH_3^+ , $N(R_u)(R_v)$, guanidino and acyl where said acyl is an acid amide or an ester;

or R_m and R_n , together, are a nitrogen protecting group, are joined in a ring
20 structure that optionally includes an additional heteroatom selected from N and O or are a chemical functional group;

R_i is OR_z , SR_z , or $N(R_z)_2$;

each R_z is, independently, H, C_1 - C_8 alkyl, C_1 - C_8 haloalkyl, $C(=NH)N(H)R_u$, $C(=O)N(H)R_u$ or $OC(=O)N(H)R_u$;

25 R_f , R_g and R_h comprise a ring system having from about 4 to about 7 carbon atoms or having from about 3 to about 6 carbon atoms and 1 or 2 heteroatoms wherein said heteroatoms are selected from oxygen, nitrogen and sulfur and wherein said ring system is aliphatic, unsaturated aliphatic, aromatic, or saturated or unsaturated heterocyclic;

R_j is alkyl or haloalkyl having 1 to about 10 carbon atoms, alkenyl having 2 to
30 about 10 carbon atoms, alkynyl having 2 to about 10 carbon atoms, aryl having 6 to about 14 carbon atoms, $N(R_k)(R_m)$ OR_k , halo, SR_k or CN;

m_a is 1 to about 10;

each mb is, independently, 0 or 1;

mc is 0 or an integer from 1 to 10;

md is an integer from 1 to 10;

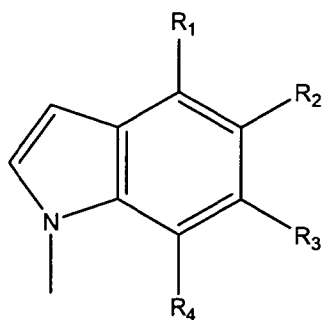
me is from 0, 1 or 2; and

5 provided that when mc is 0, md is greater than 1.

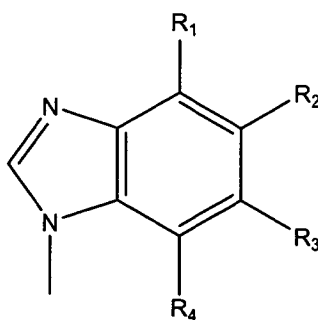
In some embodiments, each of j and r are at least 2. In some further embodiments, T₃, T₄ and T₅ each comprise a nucleotide having one of an eastern or southern conformation. In some embodiments, at least one of T₃, T₄ and T₅ comprise a 2'-fluoro-arabinonucleotide. In some embodiments, each of T₃, T₄ and T₅ comprise a 2'-fluoro-
10 arabinonucleotide.

In some embodiments, each of n and p are 0.

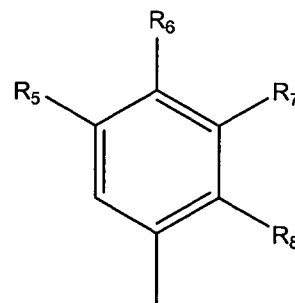
In some embodiments, each T₂ comprises a nucleotide having a 2'-modification; each of j and r are at least 2; and T₃, T₄ and T₅ each comprise a nucleotide having one of an eastern or southern conformation. In some each embodiments, T₃, T₄ and T₅
15 each comprise a nucleotide having an eastern conformation. In some embodiments, at least one of T₃, T₄ and T₅ comprise a 2'-fluoro-arabinonucleotide, an abasic nucleotide, a THF nucleoside, or a nucleotide having a nucleobase selected from Formulas I, II, and III:



I



II



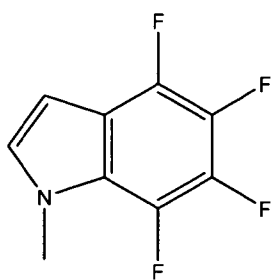
III

20

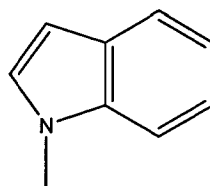
wherein:

each of R₁₋₈ is independently selected from H, halogen and C₁₋₃ alkyl. In some embodiments, R₁₋₈ is independently selected from fluorine and methyl. In some embodiments, nucleobase is selected from Formulas IV, V or VI:

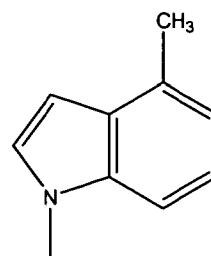
25



IV

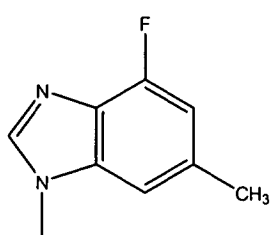


V

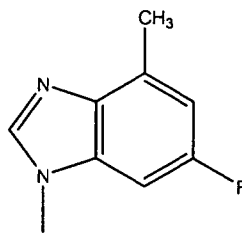


VI

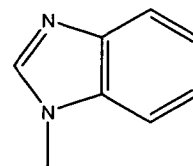
or Formulas VII, VIII, IX, X or XI:



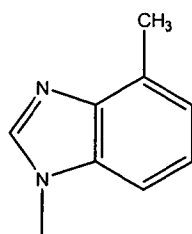
VII



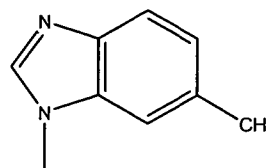
VIII



IX

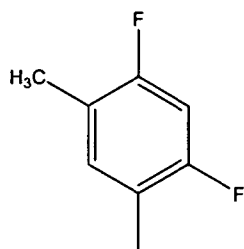


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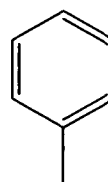


XI

5 or Formulas XII or XIII:



XII



XIII

In some embodiments, j and r are each from 2 to 5, and m is 10 to 16. In some
10 embodiments, j is 2, r is 2 and m is 14-18. In some embodiments, j is 2, r is 2 and m is 16.
In some embodiments, j is 4, r is 4 and m is 10-14. In some embodiments, j is 4, r is 4 and

m is 12. In some embodiments, j is 5, r is 5 and m is 8-12. In some embodiments, j is 5, r is 5 and m is 10.

In some embodiments, the invention provides methods of increasing one of the rate of cleavage or the position of cleavage of a target RNA by RNase H comprising:

5 selecting an oligonucleotide having an RNase H cleaving region and a non-RNase H cleaving region;

selecting a transitional nucleotide capable of modulating transfer of the helical conformation characteristic of an oligonucleotide bound to its 3'hydroxy to an oligonucleotide bound to its 5' hydroxyl;

10 interspacing said transitional nucleotide in said oligonucleotide positioned between said RNase H cleaving region and said non-RNase H cleaving region; and

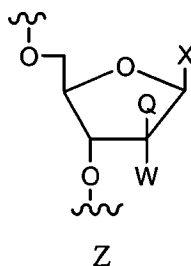
binding said oligonucleotide to said target RNA in the presence of RNase H.

In some embodiments, the oligonucleotide has the Formula:

$(T_2)_j-(T_3)_k-(T_1)_m-(T_4)_n-(T_1)_p-(T_5)_q-(T_2)_r$.

15 In some embodiments, the transitional nucleotide bears a nucleobase having one of the structures IV-XIII, *supra*.

In some embodiments, the transitional nucleotide has the Formula Z:



20 wherein:

X is selected from hydrogen, substituted or unsubstituted indolyl, substituted or unsubstituted benzimidazolyl, substituted or unsubstituted purinyl, or substituted or unsubstituted pyrimidinyl;

Q is selected from hydrogen or halogen;

25 W is selected from hydrogen or a 2'-substituent.

In some embodiments, the transitional nucleotide is a 2'-fluoro-arabinonucleotide.

In some embodiments, the invention provides antisense oligonucleotides, comprising:

at least 2 conformationally different regions with a junction between each of said conformationally different regions, wherein each junction comprises at least one transitional nucleotide capable of modulating transfer of the helical conformation characteristic of an oligonucleotide bound to its 3'hydroxy to an oligonucleotide bound to its 5' hydroxyl.

In some embodiments, the conformationally different regions each comprise at least two nucleotides. In further embodiments, the nucleotides of each conformationally different region each possess the same type of sugar conformation. In some embodiments, the type of sugar conformation is selected from Eastern, Northern and Southern.

In some embodiments, the transitional nucleotide is a base-modified nucleotide or a sugar-modified nucleotide.

In some embodiments, base-modified nucleotide has the Formula Z.

In some embodiments, the 2'-substituent is a substituted or unsubstituted aliphatic ether. In some embodiments, X is selected from Formulas I-XIII of claims 21-23. In some such embodiments, W is H, and Q is F.

Also provided are methods of inhibiting gene expression comprising contacting one or more cells, a tissue or an animal with one or more compositions of the invention.

Brief Description of the Figures

Figure 1 shows position and structure of the deoxyribonucleotide and intranucleotide linker modifications (A) Modified nucleotides containing conformationally biased sugar puckers. The northern biased modifications include: 2-thiouridine (S^2T) and 2'-fluorothymidine (2'-F). The southern biased modifications include: 2'-methylthiothymidine (2'-S-methyl), 4'-methylthymidine (4'-methyl), 3'-methylthymidine (3'-methyl), and pseudouridine. 2'-ara-fluoropyrimidine (2'-ara-Fluoro) represents the eastern biased sugar modification. (B) Structures of the modifications designed to introduce conformational flexibility into the heteroduplex. These modifications include: the propyl (C_3 '), butyl (C_4) and pentyl (C_5) hydrocarbon linkers; tetrahydrofuran (THF), abasic and ganciclovir (G_v) modifications; and the Π -stacking 2-fluoro-6-methylbenzoimidazole (2-F-6-Me-ben), 4-methylbenzoimidazole (4-Me-ben) and 2,4-difluorotoluy (2,4-F-tolyl) deoxyribonucleotides. (C) The (x) shows the position of the

modification for the respective oligodeoxyribonucleotide. The positions are numbered 5'→3' on the oligodeoxyribonucleotide.

Figure 2 shows human RNase H1 cleavage pattern and site-specific rates for modified heteroduplex substrates. (A) Polyacrylamide gel analysis of the unmodified substrate and heteroduplexes containing the 4'-methylthymidine (4'-methyl) modification at position T₉ and the 2-thiouridine (S²U) at position T₈. The substrates were incubated in the absence (Lanes 1, 7 and 13) presence of human RNase H1 for: 5 min. (Lanes 2, 8 and 14); 10 min (Lanes 3, 9 and 15); 15 min (Lanes 4, 10 and 16); 30 min. (Lanes 5, 11 and 17); and 60 min. (Lanes 6, 12 and 18). The ribonucleotide positions are numbered accordingly: the ribonucleotide opposing the modification (0); the first (-1) and second (-2) ribonucleotides 5' to the modification; and the first (+1) and second (+2) ribonucleotides 3' to the modification. (B) Site-specific cleavage rates for unmodified substrate (gray bar), the 4'-methylthymidine modification at position T₉ (black bar) and the 2'-thiothymidine at position T₈ (hashed bar). Initial rates for each site on the heteroduplex were determined as described for multiple-turnover kinetics in Material and Methods. The V₀ values are an average of three measurements with estimated errors of CV < 10%.

Figure 3 shows a model for the interaction of RNase H1 with the heteroduplex substrate at the catalytic site. The designations for ribonucleotide positions are described in Figure 2. The positions of the amino acid shown are for *E. coli* RNase H1 and the positions of the conserved amino acid are in the human enzyme are shown in brackets.

Detailed Description of the Invention

The present invention provides compounds and methods for the optimization of cleavage of RNA targets by RNase H. By determining the structure activity relationships for the interaction between the catalytic domain of human RNase H1 and the RNA/DNA heteroduplex substrate by systematically evaluating the influence of sugar conformation, it has been discovered that nucleotides minimizing bulk in the minor groove and flexibility in the catalytic area are beneficial to enzyme efficiency. Modified nucleotides were introduced into the oligodeoxyribonucleotides at the human RNase H1 preferred cleavage sites on the heteroduplex and consisted of the DNA-like southern C_{2'}-endo, RNA-like northern C_{3'}-endo and eastern O_{4'}-endo biased sugars with and without 2'-substituents

(Fig. 1A). In addition, varying degrees of conformational flexibility were introduced into the heteroduplex substrate by incorporating modified deoxyribonucleotides that Π -stack with the adjacent deoxyribonucleotides but do not form hydrogen bonds with the bases of the RNA strand, abasic deoxynucleotides, hydrocarbon intranucleotide linkers and the ganciclovir modified deoxyribonucleotide (Fig. 1B). The initial cleavage rates (V_0) as well as site-specific cleavage rates for the modified heteroduplexes were compared with the wild type DNA/RNA heteroduplex.

It has been discovered in accordance with the present invention that the incorporation of one or more transitional nucleotides in an antisense oligonucleotide can optimize the rate of cleavage of the duplex formed between the RNA target and the antisense oligonucleotide. In some embodiments, the transitional nucleotides are interspersed at the junction between regions of the antisense oligonucleotide that possess different conformation. While not wishing to be bound by a particular theory, it is believed that optimizing the helical geometry of the heteroduplex for RNase H1 cleavage can be accomplished by interspersing one or more transitional nucleotides at the junction of regions of different conformation in an antisense oligonucleotide. For example, in accordance with some embodiments of the invention, a gapmer having a plurality of regions of at least two differing conformation types can have one or more transitional nucleotides positioned at the junction of the regions.

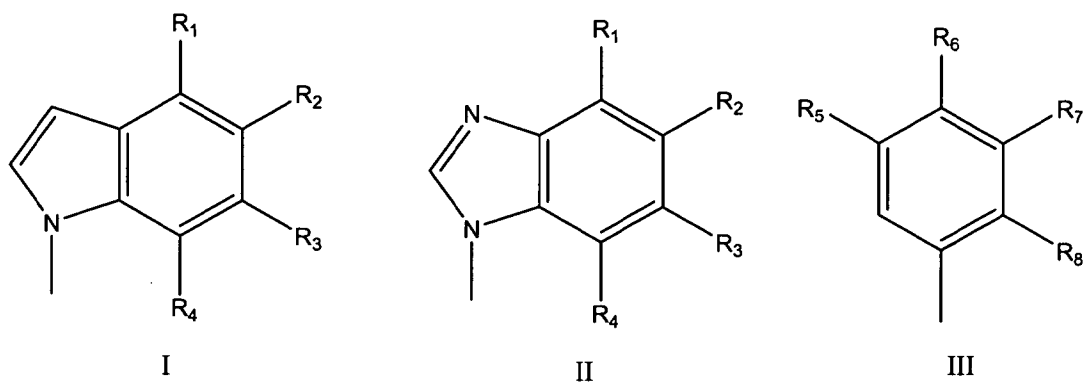
In some embodiments, the nucleotides of the “wing” regions of the gapmer can have A form geometry (e.g., northern conformation). Examples of such nucleotides are those having 2'-modifications, for example 2'-MOE. In some embodiments, the “gap” regions have H-form geometry, for example DNA nucleotides. Generally, the transitional nucleotide or nucleotides will be present at the junction of the regions, so as to impart a transition between the two regions of differing conformation.

In some embodiments, it is beneficial to minimize the lengths of the “wings” of the gapmer, and/or to further substitute 2'-substituted (e.g. MOE) nucleotides with one or more additional nuclease resistant modifications (e.g., methylphosphonate, phosphonoacetate, dangling steric blockers, etc.). In some embodiments, it also is beneficial to optimize helical conformation for cleavage, for example by use of, *inter alia*, nonn-canonical base pairs.

Because the catalytic site of bound RNase H1 is located about one full helical turn from the RNA binding site, in some embodiments, it is beneficial to have at least one transitional nucleotide located 5 to 10 nucleobases from the 3'-terminus of the antisense oligonucleotide, or 6 to 9 nucleobases from the 3'-terminus of the antisense oligonucleotide, or 7 to 8 nucleobases from the 3'-terminus of the antisense oligonucleotide. In addition to the benefit of altering the helical geometry and eliminate bulk in the minor groove, it is believed that the transitional nucleobases can correct conformational transmission from one conformational region to the next.

As used herein, the term transitional nucleotide (or "flexible nucleotide") is intended to mean a nucleotide that capable of modulating transfer of the helical conformation characteristic of an oligonucleotide bound to its 3'hydroxy to an oligonucleotide bound to its 5' hydroxyl, when the oligonucleotide is in a duplex with RNA.

Examples of such transitional nucleotides include those that having one of an eastern or southern conformation, 2'-fluoro-arabinonucleotide, abasic nucleotides, and THF nucleosides. Further examples include nucleotides having a nucleobase selected from Formulas I, II, and III:

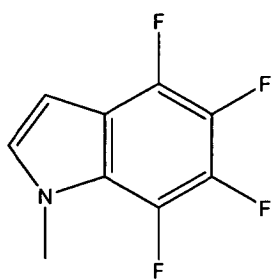


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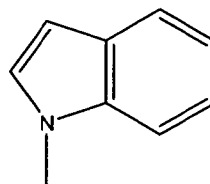
wherein:

each of R₁₋₈ is independently selected from H, halogen and C₁₋₃ alkyl. In some embodiments, R₁₋₈ is independently selected from fluorine and methyl. In some embodiments, nucleobase is selected from Formulas IV, V or VI:

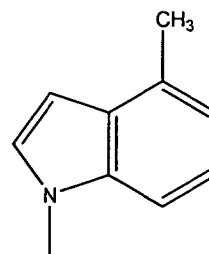
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IV

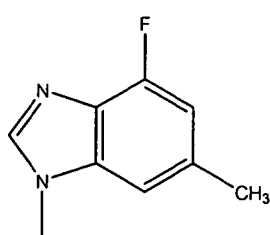


V

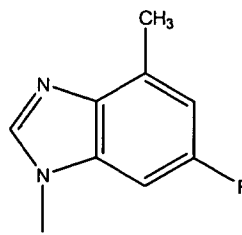


VI

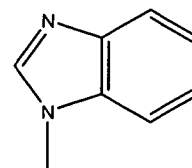
or Formulas VII, VIII, IX, X or XI:



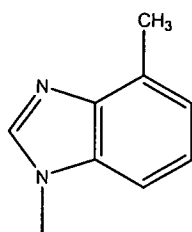
VII



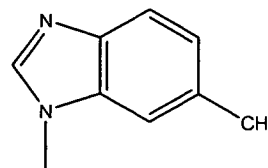
VIII



IX

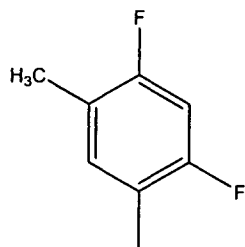


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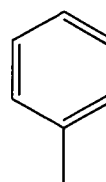


XI

5 or Formulas XII or XIII:



XII



XIII

In some embodiments, the invention provides compounds of the Formula:

10 $(T_2)_j-(T_3)_k-(T_1)_m-(T_4)_n-(T_1)_p-(T_5)_q-(T_2)_r$

wherein

each T_1 is a 2'-deoxyribonucleotide;

each T_2 is a nucleotide having a higher binding affinity for a RNA target as compared to the binding affinity of a 2'-deoxyribonucleotide for said RNA target;

each T_3 , T_4 and T_5 are transitional nucleotides;

5 j and r independently are 0 to 10, and together the sum of j and r is at least 2;

m and p independently are 1 to 20, and together the sum of m and p is at least 5;

k , n and q independently are 0 to 3, and together the sum of k , n and q is at least 1.

In some embodiments, T_2 comprises a nucleotide having a northern conformation.

In some such embodiments, T_2 comprises a nucleotide having a 2'-modification.

10 In some embodiments, j and r are each from 2 to 5, and m is 10 to 16. In some embodiments, j is 2, r is 2 and m is 14-18. In some embodiments, j is 2, r is 2 and m is 16. In some embodiments, j is 4, r is 4 and m is 10-14. In some embodiments, j is 4, r is 4 and m is 12. In some embodiments, j is 5, r is 5 and m is 8-12. In some embodiments, j is 5, r is 5 and m is 10.

15 In some embodiments, the invention provides methods of increasing one of the rate of cleavage or the position of cleavage of a target RNA by RNase H comprising:

selecting an oligonucleotide having an RNase H cleaving region and a non-RNase H cleaving region;

20 selecting a transitional nucleotide capable of modulating transfer of the helical conformation characteristic of an oligonucleotide bound to its 3'hydroxy to an oligonucleotide bound to its 5' hydroxyl;

interspacing said transitional nucleotide in said oligonucleotide positioned between said RNase H cleaving region and said non-RNase H cleaving region; and

binding said oligonucleotide to said target RNA in the presence of RNase H.

25 In some embodiments, the oligonucleotide has the Formula:

$(T_2)_j-(T_3)_k-(T_1)_m-(T_4)_n-(T_1)_p-(T_5)_q-(T_2)_r$.

In some embodiments, the transitional nucleotide bears a nucleobase having one of the structures IV-XIII, *supra*.

30 Compounds of the present invention will be useful for the modulation of gene expression. In one aspect of the present invention a targeted cell, group of cells, a tissue or an animal is contacted with a composition of the invention to effect reduction of message that can directly inhibit gene expression. In another embodiment the reduction of

message indirectly upregulates a non-targeted gene through a pathway that relates the targeted gene to a non-targeted gene. Methods and models for the regulation of genes using oligomeric compounds of the invention are illustrated in the examples.

5 In another aspect a method of inhibiting gene expression is disclosed comprising contacting one or more cells, a tissue or an animal with a compound of the invention.

Compositions of the invention modulate gene expression by hybridizing to a nucleic acid target resulting in loss of its normal function. As used herein, the term "target nucleic acid" or "nucleic acid target" is used for convenience to encompass any nucleic acid capable of being targeted including without limitation DNA, RNA (including pre-
10 mRNA and mRNA or portions thereof) transcribed from such DNA, and also cDNA derived from such RNA. In a preferred embodiment of the invention the target nucleic acid is a messenger RNA. In a further preferred embodiment the degradation of the targeted messenger RNA is facilitated by a RISC complex that is formed with oligomeric compounds of the invention. In another preferred embodiment the degradation of the
15 targeted messenger RNA is facilitated by a nuclease such as RNaseH.

The hybridization of an oligomeric compound of this invention with its target nucleic acid is generally referred to as "antisense". Consequently, the preferred mechanism in the practice of some preferred embodiments of the invention is referred to herein as "antisense inhibition." Such antisense inhibition is typically based upon
20 hydrogen bonding-based hybridization of oligonucleotide strands or segments such that at least one strand or segment is cleaved, degraded, or otherwise rendered inoperable. In this regard, it is presently preferred to target specific nucleic acid molecules and their functions for such antisense inhibition.

The functions of DNA to be interfered with can include replication and
25 transcription. Replication and transcription, for example, can be from an endogenous cellular template, a vector, a plasmid construct or otherwise. The functions of RNA to be interfered with can include functions such as translocation of the RNA to a site of protein translation, translocation of the RNA to sites within the cell which are distant from the site of RNA synthesis, translation of protein from the RNA, splicing of the RNA to yield one
30 or more RNA species, and catalytic activity or complex formation involving the RNA which may be engaged in or facilitated by the RNA. In the context of the present invention, "modulation" and "modulation of expression" mean either an increase

(stimulation) or a decrease (inhibition) in the amount or levels of a nucleic acid molecule encoding the gene, e.g., DNA or RNA. Inhibition is often the preferred form of modulation of expression and mRNA is often a preferred target nucleic acid.

The compositions and methods of the present invention are also useful in the study, characterization, validation and modulation of small non-coding RNAs. These include, but are not limited to, microRNAs (miRNA), small nuclear RNAs (snRNA), small nucleolar RNAs (snoRNA), small temporal RNAs (stRNA) and tiny non-coding RNAs (tncRNA) or their precursors or processed transcripts or their association with other cellular components.

Small non-coding RNAs have been shown to function in various developmental and regulatory pathways in a wide range of organisms, including plants, nematodes and mammals. MicroRNAs are small non-coding RNAs that are processed from larger precursors by enzymatic cleavage and inhibit translation of mRNAs. stRNAs, while processed from precursors much like miRNAs, have been shown to be involved in developmental timing regulation. Other non-coding small RNAs are involved in events as diverse as cellular splicing of transcripts, translation, transport, and chromosome organization.

As modulators of small non-coding RNA function, the compositions of the present invention find utility in the control and manipulation of cellular functions or processes such as regulation of splicing, chromosome packaging or methylation, control of developmental timing events, increase or decrease of target RNA expression levels depending on the timing of delivery into the specific biological pathway and translational or transcriptional control. In addition, the compositions of the present invention can be modified in order to optimize their effects in certain cellular compartments, such as the cytoplasm, nucleus, nucleolus or mitochondria.

The compositions of the present invention can further be used to identify components of regulatory pathways of RNA processing or metabolism as well as in screening assays or devices.

Oligomeric Compounds

In the context of the present invention, the term "oligomeric compound" refers to a polymeric structure capable of hybridizing a region of a nucleic acid molecule. This term

includes oligonucleotides, oligonucleosides, oligonucleotide analogs, oligonucleotide mimetics and combinations of these. Oligomeric compounds are routinely prepared linearly but can be joined or otherwise prepared to be circular and may also include branching. Oligomeric compounds can include double stranded constructs such as for example two strands hybridized to form double stranded compounds. The double stranded compounds can be linked or separate and can include overhangs on the ends. In general an oligomeric compound comprises a backbone of linked monomeric subunits where each linked monomeric subunit is directly or indirectly attached to a heterocyclic base moiety. Oligomeric compounds may also include monomeric subunits that are not linked to a heterocyclic base moiety thereby providing abasic sites. The linkages joining the monomeric subunits, the sugar moieties or surrogates and the heterocyclic base moieties can be independently modified giving rise to a plurality of motifs for the resulting oligomeric compounds including hemimers, gapmers and chimeras.

As is known in the art, a nucleoside is a base-sugar combination. The base portion of the nucleoside is normally a heterocyclic base moiety. The two most common classes of such heterocyclic bases are purines and pyrimidines. Nucleotides are nucleosides that further include a phosphate group covalently linked to the sugar portion of the nucleoside. For those nucleosides that include a pentofuranosyl sugar, the phosphate group can be linked to either the 2', 3' or 5' hydroxyl moiety of the sugar. In forming oligonucleotides, the phosphate groups covalently link adjacent nucleosides to one another to form a linear polymeric compound. The respective ends of this linear polymeric structure can be joined to form a circular structure by hybridization or by formation of a covalent bond, however, open linear structures are generally preferred. Within the oligonucleotide structure, the phosphate groups are commonly referred to as forming the internucleoside linkages of the oligonucleotide. The normal internucleoside linkage of RNA and DNA is a 3' to 5' phosphodiester linkage.

In the context of this invention, the term "oligonucleotide" refers to an oligomer or polymer of ribonucleic acid (RNA) or deoxyribonucleic acid (DNA). This term includes oligonucleotides composed of naturally-occurring nucleobases, sugars and covalent internucleoside linkages. The term "oligonucleotide analog" refers to oligonucleotides that have one or more non-naturally occurring portions which function in a similar manner to oligonucleotides. Such non-naturally occurring oligonucleotides are often preferred the

naturally occurring forms because of desirable properties such as, for example, enhanced cellular uptake, enhanced affinity for nucleic acid target and increased stability in the presence of nucleases.

In the context of this invention, the term "oligonucleoside" refers to a sequence of nucleosides that are joined by internucleoside linkages that do not have phosphorus atoms. Internucleoside linkages of this type include short chain alkyl, cycloalkyl, mixed heteroatom alkyl, mixed heteroatom cycloalkyl, one or more short chain heteroatomic and one or more short chain heterocyclic. These internucleoside linkages include but are not limited to siloxane, sulfide, sulfoxide, sulfone, acetyl, formacetyl, thioformacetyl, methylene formacetyl, thioformacetyl, alkenyl, sulfamate; methyleneimino, methylenehydrazino, sulfonate, sulfonamide, amide and others having mixed N, O, S and CH₂ component parts.

Representative United States patents that teach the preparation of the above oligonucleosides include, but are not limited to, U.S.: 5,034,506; 5,166,315; 5,185,444; 5,214,134; 5,216,141; 5,235,033; 5,264,562; 5,264,564; 5,405,938; 5,434,257; 5,466,677; 5,470,967; 5,489,677; 5,541,307; 5,561,225; 5,596,086; 5,602,240; 5,610,289; 5,602,240; 5,608,046; 5,610,289; 5,618,704; 5,623,070; 5,663,312; 5,633,360; 5,677,437; 5,792,608; 5,646,269 and 5,677,439, certain of which are commonly owned with this application, and each of which is herein incorporated by reference.

Further included in the present invention are oligomeric compounds such as antisense oligomeric compounds, antisense oligonucleotides, ribozymes, external guide sequence (EGS) oligonucleotides, alternate splicers, primers, probes, and other oligomeric compounds which hybridize to at least a portion of the target nucleic acid. As such, these oligomeric compounds may be introduced in the form of single-stranded, double-stranded, circular or hairpin oligomeric compounds and may contain structural elements such as internal or terminal bulges or loops. Once introduced to a system, the oligomeric compounds of the invention may elicit the action of one or more enzymes or structural proteins to effect modification of the target nucleic acid.

One non-limiting example of such an enzyme is RNase H, a cellular endonuclease which cleaves the RNA strand of an RNA:DNA duplex. It is known in the art that single-stranded antisense oligomeric compounds which are "DNA-like" elicit RNase H. Activation of RNase H, therefore, results in cleavage of the RNA target, thereby greatly

enhancing the efficiency of oligonucleotide-mediated inhibition of gene expression. Similar roles have been postulated for other ribonucleases such as those in the RNase III and ribonuclease L family of enzymes.

While the preferred form of antisense oligomeric compound is a single-stranded
5 antisense oligonucleotide, in many species the introduction of double-stranded structures, such as double-stranded RNA (dsRNA) molecules, has been shown to induce potent and specific antisense-mediated reduction of the function of a gene or its associated gene products. This phenomenon occurs in both plants and animals and is believed to have an evolutionary connection to viral defense and transposon silencing.

10 In addition to the modifications described above, the nucleosides of the oligomeric compounds of the invention can have a variety of other modification so long as these other modifications either alone or in combination with other nucleosides enhance one or more of the desired properties described above. Thus, for nucleotides that are incorporated into oligonucleotides of the invention, these nucleotides can have sugar portions that
15 correspond to naturally-occurring sugars or modified sugars. Representative modified sugars include carbocyclic or acyclic sugars, sugars having substituent groups at one or more of their 2', 3' or 4' positions and sugars having substituents in place of one or more hydrogen atoms of the sugar. Additional nucleosides amenable to the present invention having altered base moieties and or altered sugar moieties are disclosed in United States
20 Patent 3,687,808 and PCT application PCT/US89/02323.

Altered base moieties or altered sugar moieties also include other modifications consistent with the spirit of this invention. Such oligonucleotides are best described as being structurally distinguishable from, yet functionally interchangeable with, naturally occurring or synthetic wild type oligonucleotides. All such oligonucleotides are
25 comprehended by this invention so long as they function effectively to mimic the structure of a desired RNA or DNA strand. A class of representative base modifications include tricyclic cytosine analog, termed "G clamp" (Lin, *et al.*, *J. Am. Chem. Soc.* 1998, 120, 8531). This analog makes four hydrogen bonds to a complementary guanine (G) within a helix by simultaneously recognizing the Watson-Crick and Hoogsteen faces of the targeted
30 G. This G clamp modification when incorporated into phosphorothioate oligonucleotides, dramatically enhances antisense potencies in cell culture. The oligonucleotides of the

invention also can include phenoxazine-substituted bases of the type disclosed by Flanagan, *et al.*, *Nat. Biotechnol.* 1999, 17(1), 48-52.

The oligomeric compounds in accordance with this invention preferably comprise from about 8 to about 80 nucleobases (i.e. from about 8 to about 80 linked nucleosides).

5 One of ordinary skill in the art will appreciate that the invention embodies oligomeric compounds of 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, or 80 nucleobases in length.

10 In one preferred embodiment, the oligomeric compounds of the invention are 12 to 50 nucleobases in length. One having ordinary skill in the art will appreciate that this embodies oligomeric compounds of 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 nucleobases in length.

15 In another preferred embodiment, the oligomeric compounds of the invention are 15 to 30 nucleobases in length. One having ordinary skill in the art will appreciate that this embodies oligomeric compounds of 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 nucleobases in length.

Particularly preferred oligomeric compounds are oligonucleotides from about 12 to
20 about 50 nucleobases, even more preferably those comprising from about 15 to about 30 nucleobases.

Oligomeric compounds used in the compositions of the present invention can also be modified to have one or more stabilizing groups that are generally attached to one or both termini of oligomeric compounds to enhance properties such as for example nuclease
25 stability. Included in stabilizing groups are cap structures. By "cap structure or terminal cap moiety" is meant chemical modifications, which have been incorporated at either terminus of oligonucleotides (see for example Wincott et al., WO 97/26270, incorporated by reference herein). These terminal modifications protect the oligomeric compounds having terminal nucleic acid molecules from exonuclease degradation, and can help in
30 delivery and/or localization within a cell. The cap can be present at the 5'-terminus (5'-cap) or at the 3'-terminus (3'-cap) or can be present on both termini. In non-limiting examples, the 5'-cap includes inverted abasic residue (moiety), 4',5'-methylene nucleotide;

1-(beta-D-erythrofuransyl) nucleotide, 4'-thio nucleotide, carbocyclic nucleotide; 1,5-anhydrohexitol nucleotide; L-nucleotides; alpha-nucleotides; modified base nucleotide; phosphorodithioate linkage; threo-pentofuransyl nucleotide; acyclic 3',4'-seco nucleotide; acyclic 3,4-dihydroxybutyl nucleotide; acyclic 3,5-dihydroxypentyl nucleotide, 3'-3'-
5 inverted nucleotide moiety; 3'-3'-inverted abasic moiety; 3'-2'-inverted nucleotide moiety; 3'-2'-inverted abasic moiety; 1,4-butanediol phosphate; 3'-phosphoramidate; hexylphosphate; aminohexyl phosphate; 3'-phosphate; 3'-phosphorothioate; phosphorodithioate; or bridging or non-bridging methylphosphonate moiety (for more details see Wincott et al., International PCT publication No. WO 97/26270, incorporated
10 by reference herein).

Particularly preferred 3'-cap structures of the present invention include, for example 4',5'-methylene nucleotide; 1-(beta-D-erythrofuransyl) nucleotide; 4'-thio nucleotide, carbocyclic nucleotide; 5'-amino-alkyl phosphate; 1,3-diamino-2-propyl phosphate, 3-aminopropyl phosphate; 6-aminohexyl phosphate; 1,2-aminododecyl
15 phosphate; hydroxypropyl phosphate; 1,5-anhydrohexitol nucleotide; L-nucleotide; alpha-nucleotide; modified base nucleotide; phosphorodithioate; threo-pentofuransyl nucleotide; acyclic 3',4'-seco nucleotide; 3,4-dihydroxybutyl nucleotide; 3,5-dihydroxypentyl nucleotide, 5'-5'-inverted nucleotide moiety; 5'-5'-inverted abasic moiety; 5'-phosphoramidate; 5'-phosphorothioate; 1,4-butanediol phosphate; 5'-amino; bridging
20 and/or non-bridging 5'-phosphoramidate, phosphorothioate and/or phosphorodithioate, bridging or non bridging methylphosphonate and 5'-mercapto moieties (for more details see Beaucage and Tyler, 1993, Tetrahedron 49, 1925; incorporated by reference herein).

Oligomer and Monomer Modifications

25 As is known in the art, a nucleoside is a base-sugar combination. The base portion of the nucleoside is normally a heterocyclic base. The two most common classes of such heterocyclic bases are the purines and the pyrimidines. Nucleotides are nucleosides that further include a phosphate group covalently linked to the sugar portion of the nucleoside. For those nucleosides that include a pentofuransyl sugar, the phosphate group can be
30 linked to either the 2', 3' or 5' hydroxyl moiety of the sugar. In forming oligonucleotides, the phosphate groups covalently link adjacent nucleosides to one another to form a linear polymeric compound. In turn, the respective ends of this linear polymeric compound can

be further joined to form a circular compound, however, linear compounds are generally preferred. In addition, linear compounds may have internal nucleobase complementarity and may therefore fold in a manner as to produce a fully or partially double-stranded compound. Within oligonucleotides, the phosphate groups are commonly referred to as
5 forming the internucleoside linkage or in conjunction with the sugar ring the backbone of the oligonucleotide. The normal internucleoside linkage that makes up the backbone of RNA and DNA is a 3' to 5' phosphodiester linkage.

Chimeric oligomeric compounds

10 It is not necessary for all positions in a oligomeric compound to be uniformly modified, and in fact more than one of the aforementioned modifications may be incorporated in a single oligomeric compound or even at a single monomeric subunit such as a nucleoside within a oligomeric compound. The present invention also includes oligomeric compounds which are chimeric oligomeric compounds. "Chimeric" oligomeric
15 compounds or "chimeras," in the context of this invention, are oligomeric compounds containing two or more chemically distinct regions, each made up of at least one monomer unit, i.e., a nucleotide in the case of a nucleic acid based oligomer.

Chimeric oligomeric compounds typically contain at least one region modified so as to confer increased resistance to nuclease degradation, increased cellular uptake, and/or
20 increased binding affinity for the target nucleic acid. An additional region of the oligomeric compound may serve as a substrate for enzymes capable of cleaving RNA:DNA or RNA:RNA hybrids. By way of example, RNase H is a cellular endonuclease which cleaves the RNA strand of an RNA:DNA duplex. Activation of RNase H, therefore, results in cleavage of the RNA target, thereby greatly enhancing the
25 efficiency of inhibition of gene expression. Consequently, comparable results can often be obtained with shorter oligomeric compounds when chimeras are used, compared to for example phosphorothioate deoxyoligonucleotides hybridizing to the same target region. Cleavage of the RNA target can be routinely detected by gel electrophoresis and, if necessary, associated nucleic acid hybridization techniques known in the art.

30 Chimeric oligomeric compounds of the invention may be formed as composite structures of two or more oligonucleotides, oligonucleotide analogs, oligonucleosides and/or oligonucleotide mimetics as described above. Such oligomeric compounds have

also been referred to in the art as hybrids hemimers, gapmers or inverted gapmers. Representative United States patents that teach the preparation of such hybrid structures include, but are not limited to, U.S.: 5,013,830; 5,149,797; 5,220,007; 5,256,775; 5,366,878; 5,403,711; 5,491,133; 5,565,350; 5,623,065; 5,652,355; 5,652,356; and
5 5,700,922, certain of which are commonly owned with the instant application, and each of which is herein incorporated by reference in its entirety.

Oligomer Mimetics

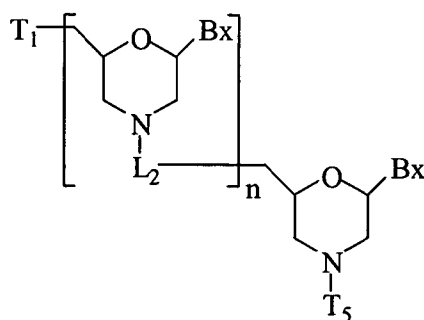
Another preferred group of oligomeric compounds amenable to the present
10 invention includes oligonucleotide mimetics. The term mimetic as it is applied to oligonucleotides is intended to include oligomeric compounds wherein only the furanose ring or both the furanose ring and the internucleotide linkage are replaced with novel groups, replacement of only the furanose ring is also referred to in the art as being a sugar surrogate. The heterocyclic base moiety or a modified heterocyclic base moiety is
15 maintained for hybridization with an appropriate target nucleic acid. One such oligomeric compound, an oligonucleotide mimetic that has been shown to have excellent hybridization properties, is referred to as a peptide nucleic acid (PNA). In PNA oligomeric compounds, the sugar-backbone of an oligonucleotide is replaced with an amide containing backbone, in particular an aminoethylglycine backbone. The
20 nucleobases are retained and are bound directly or indirectly to aza nitrogen atoms of the amide portion of the backbone. Representative United States patents that teach the preparation of PNA oligomeric compounds include, but are not limited to, U.S.: 5,539,082; 5,714,331; and 5,719,262, each of which is herein incorporated by reference. Further teaching of PNA oligomeric compounds can be found in Nielsen *et al.*, *Science*,
25 1991, 254, 1497-1500.

One oligonucleotide mimetic that has been reported to have excellent hybridization properties, is peptide nucleic acids (PNA). The backbone in PNA compounds is two or more linked aminoethylglycine units which gives PNA an amide containing backbone. The heterocyclic base moieties are bound directly or indirectly to aza nitrogen atoms of
30 the amide portion of the backbone. Representative United States patents that teach the preparation of PNA compounds include, but are not limited to, U.S.: 5,539,082; 5,714,331; and 5,719,262, each of which is herein incorporated by reference. Further

n is from 2 to about 50.

Another class of oligonucleotide mimetic that has been studied is based on linked morpholino units (morpholino nucleic acid) having heterocyclic bases attached to the morpholino ring. A number of linking groups have been reported that link the morpholino monomeric units in a morpholino nucleic acid. A preferred class of linking groups have been selected to give a non-ionic oligomeric compound. The non-ionic morpholino-based oligomeric compounds are less likely to have undesired interactions with cellular proteins. Morpholino-based oligomeric compounds are non-ionic mimics of oligonucleotides which are less likely to form undesired interactions with cellular proteins (Dwayne A. Braasch and David R. Corey, *Biochemistry*, **2002**, *41*(14), 4503-4510). Morpholino-based oligomeric compounds are disclosed in United States Patent 5,034,506, issued July 23, 1991. The morpholino class of oligomeric compounds have been prepared having a variety of different linking groups joining the monomeric subunits.

Morpholino nucleic acids have been prepared having a variety of different linking groups (L_2) joining the monomeric subunits. The basic formula is shown below:



wherein

T_1 is hydroxyl or a protected hydroxyl;

T_5 is hydrogen or a phosphate or phosphate derivative;

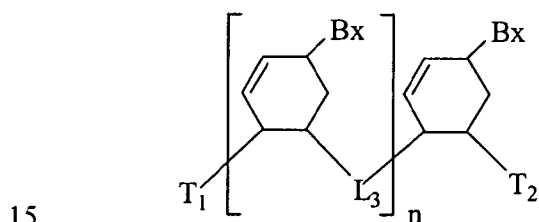
L_2 is a linking group; and

n is from 2 to about 50.

A further class of oligonucleotide mimetic is referred to as cyclohexenyl nucleic acids (CeNA). The furanose ring normally present in an DNA/RNA molecule is replaced

with a cyclohexenyl ring. CeNA DMT protected phosphoramidite monomers have been prepared and used for oligomeric compound synthesis following classical phosphoramidite chemistry. Fully modified CeNA oligomeric compounds and oligonucleotides having specific positions modified with CeNA have been prepared and studied (see Wang *et al.*,
5 *J. Am. Chem. Soc.*, **2000**, *122*, 8595-8602). In general the incorporation of CeNA monomers into a DNA chain increases its stability of a DNA/RNA hybrid. CeNA oligoadenylates formed complexes with RNA and DNA complements with similar stability to the native complexes. The study of incorporating CeNA structures into natural nucleic acid structures was shown by NMR and circular dichroism to proceed with easy
10 conformational adaptation. Furthermore the incorporation of CeNA into a sequence targeting RNA was stable to serum and able to activate E. Coli RNase resulting in cleavage of the target RNA strand.

The general formula of CeNA is shown below:



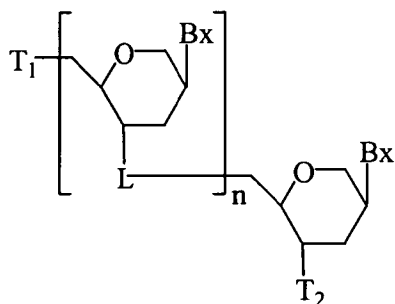
wherein

each Bx is a heterocyclic base moiety;

T₁ is hydroxyl or a protected hydroxyl; and

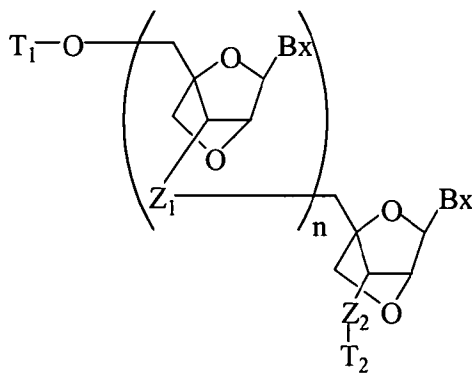
20 T₂ is hydroxyl or a protected hydroxyl.

Another class of oligonucleotide mimetic (anhydrohexitol nucleic acid) can be prepared from one or more anhydrohexitol nucleosides (see, Wouters and Herdewijn, *Bioorg. Med. Chem. Lett.*, **1999**, *9*, 1563-1566) and would have the general formula:



A further preferred modification includes Locked Nucleic Acids (LNAs) in which the 2'-hydroxyl group is linked to the 4' carbon atom of the sugar ring thereby forming a 2'-C,4'-C-oxymethylene linkage thereby forming a bicyclic sugar moiety. The linkage is preferably a methylene $(-\text{CH}_2-)_n$ group bridging the 2' oxygen atom and the 4' carbon atom wherein n is 1 or 2 (Singh et al., Chem. Commun., 1998, 4, 455-456). LNA and LNA analogs display very high duplex thermal stabilities with complementary DNA and RNA ($T_m = +3$ to $+10$ C), stability towards 3'-exonucleolytic degradation and good solubility properties. The basic structure of LNA showing the bicyclic ring system is shown below:

10



The conformations of LNAs determined by 2D NMR spectroscopy have shown that the locked orientation of the LNA nucleotides, both in single-stranded LNA and in duplexes, constrains the phosphate backbone in such a way as to introduce a higher population of the N-type conformation (Petersen et al., J. Mol. Recognit., 2000, 13, 44-53). These conformations are associated with improved stacking of the nucleobases (Wengel et al., Nucleosides Nucleotides, 1999, 18, 1365-1370).

LNA has been shown to form exceedingly stable LNA:LNA duplexes (Koshkin et al., J. Am. Chem. Soc., 1998, 120, 13252-13253). LNA:LNA hybridization was shown to

20

be the most thermally stable nucleic acid type duplex system, and the RNA-mimicking character of LNA was established at the duplex level. Introduction of 3 LNA monomers (T or A) significantly increased melting points ($T_m = +15/+11$) toward DNA complements. The universality of LNA-mediated hybridization has been stressed by the
5 formation of exceedingly stable LNA:LNA duplexes. The RNA-mimicking of LNA was reflected with regard to the N-type conformational restriction of the monomers and to the secondary structure of the LNA:RNA duplex.

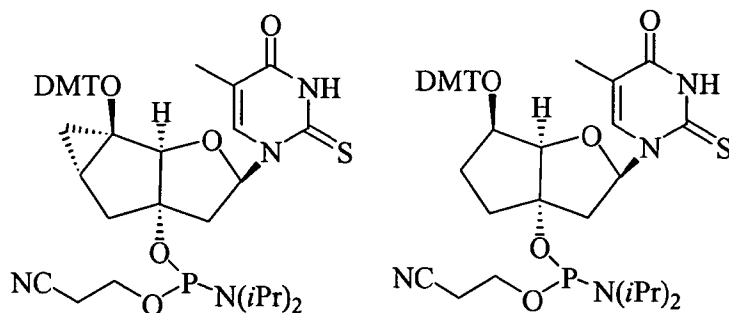
LNAs also form duplexes with complementary DNA, RNA or LNA with high thermal affinities. Circular dichroism (CD) spectra show that duplexes involving fully
10 modified LNA (esp. LNA:RNA) structurally resemble an A-form RNA:RNA duplex. Nuclear magnetic resonance (NMR) examination of an LNA:DNA duplex confirmed the 3'-endo conformation of an LNA monomer. Recognition of double-stranded DNA has also been demonstrated suggesting strand invasion by LNA. Studies of mismatched sequences show that LNAs obey the Watson-Crick base pairing rules with generally
15 improved selectivity compared to the corresponding unmodified reference strands.

Novel types of LNA-oligomeric compounds, as well as the LNAs, are useful in a wide range of diagnostic and therapeutic applications. Among these are antisense applications, PCR applications, strand-displacement oligomers, substrates for nucleic acid polymerases and generally as nucleotide based drugs.
20 Potent and nontoxic antisense oligonucleotides containing LNAs have been described (Wahlestedt et al., Proc. Natl. Acad. Sci. U. S. A., 2000, 97, 5633-5638.) The authors have demonstrated that LNAs confer several desired properties to antisense agents. LNA/DNA copolymers were not degraded readily in blood serum and cell extracts. LNA/DNA copolymers exhibited potent antisense activity in assay systems as disparate as
25 G-protein-coupled receptor signaling in living rat brain and detection of reporter genes in *Escherichia coli*. Lipofectin-mediated efficient delivery of LNA into living human breast cancer cells has also been accomplished.

The synthesis and preparation of the LNA monomers adenine, cytosine, guanine, 5-methyl-cytosine, thymine and uracil, along with their oligomerization, and nucleic acid
30 recognition properties have been described (Koshkin et al., Tetrahedron, 1998, 54, 3607-3630). LNAs and preparation thereof are also described in WO 98/39352 and WO 99/14226.

The first analogs of LNA, phosphorothioate-LNA and 2'-thio-LNAs, have also been prepared (Kumar et al., *Bioorg. Med. Chem. Lett.*, 1998, 8, 2219-2222). Preparation of locked nucleoside analogs containing oligodeoxyribonucleotide duplexes as substrates for nucleic acid polymerases has also been described (Wengel et al., PCT International Application WO 98-DK393 19980914). Furthermore, synthesis of 2'-amino-LNA, a novel conformationally restricted high-affinity oligonucleotide analog with a handle has been described in the art (Singh et al., *J. Org. Chem.*, 1998, 63, 10035-10039). In addition, 2'-Amino- and 2'-methylamino-LNA's have been prepared and the thermal stability of their duplexes with complementary RNA and DNA strands has been previously reported.

Further oligonucleotide mimetics have been prepared to include bicyclic and tricyclic nucleoside analogs having the formulas (amidite monomers shown):

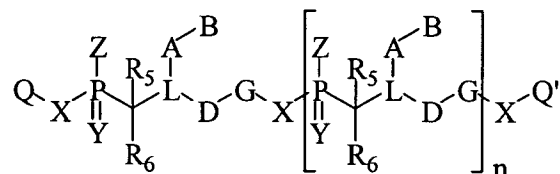


(see Steffens et al., *Helv. Chim. Acta*, 1997, 80, 2426-2439; Steffens et al., *J. Am. Chem. Soc.*, 1999, 121, 3249-3255; and Renneberg et al., *J. Am. Chem. Soc.*, 2002, 124, 5993-6002). These modified nucleoside analogs have been oligomerized using the phosphoramidite approach and the resulting oligomeric compounds containing tricyclic nucleoside analogs have shown increased thermal stabilities (*T_m*'s) when hybridized to DNA, RNA and itself. Oligomeric compounds containing bicyclic nucleoside analogs have shown thermal stabilities approaching that of DNA duplexes.

Another class of oligonucleotide mimetic is referred to as phosphonomonoester nucleic acids incorporate a phosphorus group in a backbone the backbone. This class of oligonucleotide mimetic is reported to have useful physical and biological and pharmacological properties in the areas of inhibiting gene expression (antisense

oligonucleotides, ribozymes, sense oligonucleotides and triplex-forming oligonucleotides), as probes for the detection of nucleic acids and as auxiliaries for use in molecular biology.

The general formula (for definitions of Markush variables see: United States Patents 5,874,553 and 6,127,346 herein incorporated by reference in their entirety) is shown below.



Another oligonucleotide mimetic has been reported wherein the furanosyl ring has been replaced by a cyclobutyl moiety.

Modified Internucleoside Linkages

Specific examples of preferred antisense oligomeric compounds useful in this invention include oligonucleotides containing modified e.g. non-naturally occurring internucleoside linkages. As defined in this specification, oligonucleotides having modified internucleoside linkages include internucleoside linkages that retain a phosphorus atom and internucleoside linkages that do not have a phosphorus atom. For the purposes of this specification, and as sometimes referenced in the art, modified oligonucleotides that do not have a phosphorus atom in their internucleoside backbone can also be considered to be oligonucleosides.

In the *C. elegans* system, modification of the internucleotide linkage (phosphorothioate) did not significantly interfere with RNAi activity. Based on this observation, it is suggested that certain preferred oligomeric compounds of the invention can also have one or more modified internucleoside linkages. A preferred phosphorus containing modified internucleoside linkage is the phosphorothioate internucleoside linkage.

Preferred modified oligonucleotide backbones containing a phosphorus atom therein include, for example, phosphorothioates, chiral phosphorothioates, phosphorodithioates, phosphotriesters, aminoalkylphosphotriesters, methyl and other alkyl phosphonates including 3'-alkylene phosphonates, 5'-alkylene phosphonates and chiral

phosphonates, phosphinates, phosphoramidates including 3'-amino phosphoramidate and aminoalkylphosphoramidates, thionophosphoramidates, thionoalkylphosphonates, thionoalkylphosphotriesters, selenophosphates and boranophosphates having normal 3'-5' linkages, 2'-5' linked analogs of these, and those having inverted polarity wherein one or more internucleotide linkages is a 3' to 3', 5' to 5' or 2' to 2' linkage. Preferred oligonucleotides having inverted polarity comprise a single 3' to 3' linkage at the 3'-most internucleotide linkage i.e. a single inverted nucleoside residue which may be abasic (the nucleobase is missing or has a hydroxyl group in place thereof). Various salts, mixed salts and free acid forms are also included.

Representative United States patents that teach the preparation of the above phosphorus-containing linkages include, but are not limited to, U.S.: 3,687,808; 4,469,863; 4,476,301; 5,023,243; 5,177,196; 5,188,897; 5,264,423; 5,276,019; 5,278,302; 5,286,717; 5,321,131; 5,399,676; 5,405,939; 5,453,496; 5,455,233; 5,466,677; 5,476,925; 5,519,126; 5,536,821; 5,541,306; 5,550,111; 5,563,253; 5,571,799; 5,587,361; 5,194,599; 5,565,555; 5,527,899; 5,721,218; 5,672,697 and 5,625,050, certain of which are commonly owned with this application, and each of which is herein incorporated by reference.

In more preferred embodiments of the invention, oligomeric compounds have one or more phosphorothioate and/or heteroatom internucleoside linkages, in particular -CH₂-NH-O-CH₂-, -CH₂-N(CH₃)-O-CH₂- [known as a methylene (methylimino) or MMI backbone], -CH₂-O-N(CH₃)-CH₂-, -CH₂-N(CH₃)-N(CH₃)-CH₂- and -O-N(CH₃)-CH₂-CH₂- [wherein the native phosphodiester internucleotide linkage is represented as -O-P(=O)(OH)-O-CH₂-]. The MMI type internucleoside linkages are disclosed in the above referenced U.S. patent 5,489,677. Preferred amide internucleoside linkages are disclosed in the above referenced U.S. patent 5,602,240.

Preferred modified oligonucleotide backbones that do not include a phosphorus atom therein have backbones that are formed by short chain alkyl or cycloalkyl internucleoside linkages, mixed heteroatom and alkyl or cycloalkyl internucleoside linkages, or one or more short chain heteroatomic or heterocyclic internucleoside linkages. These include those having morpholino linkages (formed in part from the sugar portion of a nucleoside); siloxane backbones; sulfide, sulfoxide and sulfone backbones; formacetyl and thioformacetyl backbones; methylene formacetyl and thioformacetyl backbones;

riboacetyl backbones; alkene containing backbones; sulfamate backbones; methyleneimino and methylenediazino backbones; sulfonate and sulfonamide backbones; amide backbones; and others having mixed N, O, S and CH₂ component parts.

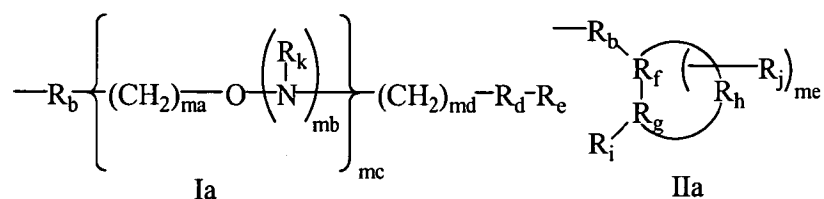
Representative United States patents that teach the preparation of the above
5 oligonucleosides include, but are not limited to, U.S.: 5,034,506; 5,166,315; 5,185,444; 5,214,134; 5,216,141; 5,235,033; 5,264,562; 5,264,564; 5,405,938; 5,434,257; 5,466,677; 5,470,967; 5,489,677; 5,541,307; 5,561,225; 5,596,086; 5,602,240; 5,610,289; 5,602,240; 5,608,046; 5,610,289; 5,618,704; 5,623,070; 5,663,312; 5,633,360; 5,677,437; 5,792,608; 5,646,269 and 5,677,439, certain of which are commonly owned with this application, and
10 each of which is herein incorporated by reference.

Modified sugars

Oligomeric compounds of the invention may also contain one or more substituted sugar moieties. Preferred oligomeric compounds comprise a sugar substituent group
15 selected from: OH; F; O-, S-, or N-alkyl; O-, S-, or N-alkenyl; O-, S- or N-alkynyl; or O-alkyl-O-alkyl, wherein the alkyl, alkenyl and alkynyl may be substituted or unsubstituted C₁ to C₁₀ alkyl or C₂ to C₁₀ alkenyl and alkynyl. Particularly preferred are O[(CH₂)_nO]_m-CH₃, O(CH₂)_nOCH₃, O(CH₂)_nNH₂, O(CH₂)_nCH₃, O(CH₂)_nONH₂, and O(CH₂)_nON-[(CH₂)_nCH₃]₂, where n and m are from 1 to about 10. Other preferred oligonucleotides
20 comprise a sugar substituent group selected from: C₁ to C₁₀ lower alkyl, substituted lower alkyl, alkenyl, alkynyl, alkaryl, aralkyl, O-alkaryl or O-aralkyl, SH, SCH₃, OCN, Cl, Br, CN, CF₃, OCF₃, SOCH₃, SO₂CH₃, ONO₂, NO₂, N₃, NH₂, heterocycloalkyl, heterocycloalkaryl, aminoalkylamino, polyalkylamino, substituted silyl, an RNA cleaving group, a reporter group, an intercalator, a group for improving the pharmacokinetic properties of an
25 oligonucleotide, or a group for improving the pharmacodynamic properties of an oligonucleotide, and other substituents having similar properties. A preferred modification includes 2'-methoxyethoxy (2'-O-CH₂CH₂OCH₃, also known as 2'-O-(2-methoxyethyl) or 2'-MOE) (Martin *et al.*, *Helv. Chim. Acta*, **1995**, *78*, 486-504) i.e., an alkoxyalkoxy group. A further preferred modification includes 2'-dimethylamino-oxyethoxy, i.e., a O(CH₂)₂ON(CH₃)₂ group, also known as 2'-DMAOE, as described in
30 examples hereinbelow, and 2'-dimethylaminoethoxyethoxy (also known in the art as 2'-O-dimethyl-amino-ethoxy-ethyl or 2'-DMAEOE), i.e., 2'-O-CH₂OCH₂N(CH₃)₂.

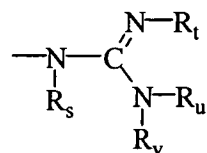
Other preferred sugar substituent groups include methoxy (-O-CH₃), aminopropoxy (-OCH₂CH₂CH₂NH₂), allyl (-CH₂-CH=CH₂), -O-allyl (-O-CH₂-CH=CH₂) and fluoro (F). 2'-Sugar substituent groups may be in the arabino (up) position or ribo (down) position. A preferred 2'-arabino modification is 2'-F. Similar modifications may also be made at other positions on the oligomeric compound, particularly the 3' position of the sugar on the 3' terminal nucleoside or in 2'-5' linked oligonucleotides and the 5' position of 5' terminal nucleotide. Oligomeric compounds may also have sugar mimetics such as cyclobutyl moieties in place of the pentofuranosyl sugar. Representative United States patents that teach the preparation of such modified sugar structures include, but are not limited to, U.S.: 4,981,957; 5,118,800; 5,319,080; 5,359,044; 5,393,878; 5,446,137; 5,466,786; 5,514,785; 5,519,134; 5,567,811; 5,576,427; 5,591,722; 5,597,909; 5,610,300; 5,627,053; 5,639,873; 5,646,265; 5,658,873; 5,670,633; 5,792,747; and 5,700,920, certain of which are commonly owned with the instant application, and each of which is herein incorporated by reference in its entirety.

Further representative sugar substituent groups include groups of formula I_a or II_a:



wherein:

- R_b is O, S or NH;
- R_d is a single bond, O, S or C(=O);
- R_e is C₁-C₁₀ alkyl, N(R_k)(R_m), N(R_k)(R_n), N=C(R_p)(R_q), N=C(R_p)(R_r) or has formula III_a;



III_a

- R_p and R_q are each independently hydrogen or C₁-C₁₀ alkyl;
- R_r is -R_x-R_y;

each R_s , R_t , R_u and R_v is, independently, hydrogen, $C(O)R_w$, substituted or unsubstituted C_1 - C_{10} alkyl, substituted or unsubstituted C_2 - C_{10} alkenyl, substituted or unsubstituted C_2 - C_{10} alkynyl, alkylsulfonyl, arylsulfonyl, a chemical functional group or a conjugate group, wherein the substituent groups are selected from hydroxyl, amino,
5 alkoxy, carboxy, benzyl, phenyl, nitro, thiol, thioalkoxy, halogen, alkyl, aryl, alkenyl and alkynyl;

or optionally, R_u and R_v , together form a phthalimido moiety with the nitrogen atom to which they are attached;

each R_w is, independently, substituted or unsubstituted C_1 - C_{10} alkyl,
10 trifluoromethyl, cyanoethoxy, methoxy, ethoxy, t-butoxy, allyloxy, 9-fluorenylmethoxy, 2-(trimethylsilyl)-ethoxy, 2,2,2-trichloroethoxy, benzyloxy, butyryl, iso-butyryl, phenyl or aryl;

R_k is hydrogen, a nitrogen protecting group or $-R_x-R_y$;

R_p is hydrogen, a nitrogen protecting group or $-R_x-R_y$;

15 R_x is a bond or a linking moiety;

R_y is a chemical functional group, a conjugate group or a solid support medium;

each R_m and R_n is, independently, H, a nitrogen protecting group, substituted or unsubstituted C_1 - C_{10} alkyl, substituted or unsubstituted C_2 - C_{10} alkenyl, substituted or unsubstituted C_2 - C_{10} alkynyl, wherein the substituent groups are selected from hydroxyl,
20 amino, alkoxy, carboxy, benzyl, phenyl, nitro, thiol, thioalkoxy, halogen, alkyl, aryl, alkenyl, alkynyl; NH_3^+ , $N(R_u)(R_v)$, guanidino and acyl where the acyl is an acid amide or an ester;

or R_m and R_n , together, are a nitrogen protecting group, are joined in a ring structure that optionally includes an additional heteroatom selected from N and O or are a
25 chemical functional group;

R_i is OR_z , SR_z , or $N(R_z)_2$;

each R_z is, independently, H, C_1 - C_8 alkyl, C_1 - C_8 haloalkyl, $C(=NH)N(H)R_u$, $C(=O)N(H)R_u$ or $OC(=O)N(H)R_u$;

R_f , R_g and R_h comprise a ring system having from about 4 to about 7 carbon atoms
30 or having from about 3 to about 6 carbon atoms and 1 or 2 heteroatoms wherein said heteroatoms are selected from oxygen, nitrogen and sulfur and wherein said ring system is aliphatic, unsaturated aliphatic, aromatic, or saturated or unsaturated heterocyclic;

R_j is alkyl or haloalkyl having 1 to about 10 carbon atoms, alkenyl having 2 to about 10 carbon atoms, alkynyl having 2 to about 10 carbon atoms, aryl having 6 to about 14 carbon atoms, $N(R_k)(R_m)OR_k$, halo, SR_k or CN ;

m_a is 1 to about 10;

5 each m_b is, independently, 0 or 1;

m_c is 0 or an integer from 1 to 10;

m_d is an integer from 1 to 10;

m_e is from 0, 1 or 2; and

provided that when m_c is 0, m_d is greater than 1.

10 Representative substituent groups of Formula I are disclosed in United States Patent Application Serial No. 09/130,973, filed August 7, 1998, entitled "Capped 2'-Oxyethoxy Oligonucleotides," hereby incorporated by reference in its entirety.

Representative cyclic substituent groups of Formula II are disclosed in United States Patent Application Serial No. 09/123,108, filed July 27, 1998, entitled "RNA
15 Targeted 2'-Oligomeric compounds that are Conformationally Preorganized," hereby incorporated by reference in its entirety.

Particularly preferred sugar substituent groups include $O[(CH_2)_nO]_mCH_3$, $O(CH_2)_nOCH_3$, $O(CH_2)_nNH_2$, $O(CH_2)_nCH_3$, $O(CH_2)_nONH_2$, and $O(CH_2)_nON[(CH_2)_nCH_3]_2$, where n and m are from 1 to about 10.

20 Representative guanidino substituent groups that are shown in formula III and IV are disclosed in co-owned United States Patent Application 09/349,040, entitled "Functionalized Oligomers", filed July 7, 1999, hereby incorporated by reference in its entirety.

Representative acetamido substituent groups are disclosed in United States Patent
25 6,147,200 which is hereby incorporated by reference in its entirety.

Representative dimethylaminoethoxyethyl substituent groups are disclosed in International Patent Application PCT/US99/17895, entitled "2'-O-Dimethylaminoethoxyethyl-Oligomeric compounds", filed August 6, 1999, hereby incorporated by reference
in its entirety.

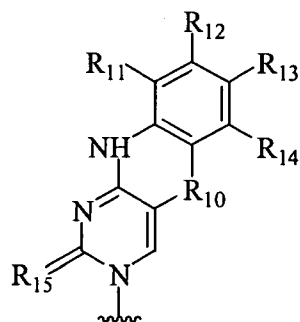
Modified Nucleobases/Naturally occurring nucleobases

Oligomeric compounds may also include nucleobase (often referred to in the art simply as "base" or "heterocyclic base moiety") modifications or substitutions. As used herein, "unmodified" or "natural" nucleobases include the purine bases adenine (A) and guanine (G), and the pyrimidine bases thymine (T), cytosine (C) and uracil (U). Modified nucleobases also referred herein as heterocyclic base moieties include other synthetic and natural nucleobases such as 5-methylcytosine (5-me-C), 5-hydroxymethyl cytosine, xanthine, hypoxanthine, 2-aminoadenine, 6-methyl and other alkyl derivatives of adenine and guanine, 2-propyl and other alkyl derivatives of adenine and guanine, 2-thiouracil, 2-thiothymine and 2-thiocytosine, 5-halouracil and cytosine, 5-propynyl ($-C\equiv C-CH_3$) uracil and cytosine and other alkynyl derivatives of pyrimidine bases, 6-azo uracil, cytosine and thymine, 5-uracil (pseudouracil), 4-thiouracil, 8-halo, 8-amino, 8-thiol, 8-thioalkyl, 8-hydroxyl and other 8-substituted adenines and guanines, 5-halo particularly 5-bromo, 5-trifluoromethyl and other 5-substituted uracils and cytosines, 7-methylguanine and 7-methyladenine, 2-F-adenine, 2-amino-adenine, 8-azaguanine and 8-azaadenine, 7-deazaguanine and 7-deazaadenine and 3-deazaguanine and 3-deazaadenine.

Heterocyclic base moieties may also include those in which the purine or pyrimidine base is replaced with other heterocycles, for example 7-deaza-adenine, 7-deazaguanosine, 2-aminopyridine and 2-pyridone. Further nucleobases include those disclosed in United States Patent No. 3,687,808, those disclosed in *The Concise Encyclopedia Of Polymer Science And Engineering*, pages 858-859, Kroschwitz, J.I., ed. John Wiley & Sons, 1990, those disclosed by Englisch et al., *Angewandte Chemie*, International Edition, 1991, 30, 613, and those disclosed by Sanghvi, Y.S., Chapter 15, *Antisense Research and Applications*, pages 289-302, Crooke, S.T. and Lebleu, B., ed., CRC Press, 1993. Certain of these nucleobases are particularly useful for increasing the binding affinity of the oligomeric compounds of the invention. These include 5-substituted pyrimidines, 6-azapyrimidines and N-2, N-6 and O-6 substituted purines, including 2-aminopropyladenine, 5-propynyluracil and 5-propynylcytosine. 5-methylcytosine substitutions have been shown to increase nucleic acid duplex stability by 0.6-1.2°C (Sanghvi, Y.S., Crooke, S.T. and Lebleu, B., eds., *Antisense Research and Applications*, CRC Press, Boca Raton, 1993, pp. 276-278) and are presently preferred base

substitutions, even more particularly when combined with 2'-O-methoxyethyl sugar modifications.

Oligomeric compounds of the present invention can also include polycyclic heterocyclic compounds in place of one or more heterocyclic base moieties. A number of tricyclic heterocyclic compounds have been previously reported. These compounds are routinely used in antisense applications to increase the binding properties of the modified strand to a target strand. The most studied modifications are targeted to guanosines hence they have been termed G-clamps or cytidine analogs. Many of these polycyclic heterocyclic compounds have the general formula:



Representative cytosine analogs that make 3 hydrogen bonds with a guanosine in a second strand include 1,3-diazaphenoxazine-2-one ($R_{10} = O$, $R_{11} - R_{14} = H$) [Kurchavov, *et al.*, *Nucleosides and Nucleotides*, 1997, 16, 1837-1846], 1,3-diazaphenothiazine-2-one ($R_{10} = S$, $R_{11} - R_{14} = H$), [Lin, K.-Y.; Jones, R. J.; Matteucci, M. J. *Am. Chem. Soc.* 1995, 117, 3873-3874] and 6,7,8,9-tetrafluoro-1,3-diazaphenoxazine-2-one ($R_{10} = O$, $R_{11} - R_{14} = F$) [Wang, J.; Lin, K.-Y., Matteucci, M. *Tetrahedron Lett.* 1998, 39, 8385-8388]. Incorporated into oligonucleotides these base modifications were shown to hybridize with complementary guanine and the latter was also shown to hybridize with adenine and to enhance helical thermal stability by extended stacking interactions (also see U.S. Patent Application entitled "Modified Peptide Nucleic Acids" filed May 24, 2002, Serial number 10/155,920; and U.S. Patent Application entitled "Nuclease Resistant Chimeric Oligonucleotides" filed May 24, 2002, Serial number 10/013,295, both of which are commonly owned with this application and are herein incorporated by reference in their entirety).

Further helix-stabilizing properties have been observed when a cytosine analog/substitute has an aminoethoxy moiety attached to the rigid 1,3-diazaphenoxazine-2-one scaffold ($R_{10} = O$, $R_{11} = -O-(CH_2)_2-NH_2$, $R_{12-14} = H$) [Lin, K.-Y.; Matteucci, M. J. Am. Chem. Soc. 1998, 120, 8531-8532]. Binding studies demonstrated that a single
5 incorporation could enhance the binding affinity of a model oligonucleotide to its complementary target DNA or RNA with a ΔT_m of up to 18° relative to 5-methyl cytosine ($dC5^{me}$), which is the highest known affinity enhancement for a single modification, yet. On the other hand, the gain in helical stability does not compromise the specificity of the oligonucleotides. The T_m data indicate an even greater discrimination between the perfect
10 match and mismatched sequences compared to $dC5^{me}$. It was suggested that the tethered amino group serves as an additional hydrogen bond donor to interact with the Hoogsteen face, namely the O6, of a complementary guanine thereby forming 4 hydrogen bonds. This means that the increased affinity of G-clamp is mediated by the combination of extended base stacking and additional specific hydrogen bonding.

15 Further tricyclic heterocyclic compounds and methods of using them that are amenable to the present invention are disclosed in United States Patent Serial Number 6,028,183, which issued on May 22, 2000, and United States Patent Serial Number 6,007,992, which issued on December 28, 1999, the contents of both are commonly assigned with this application and are incorporated herein in their entirety.

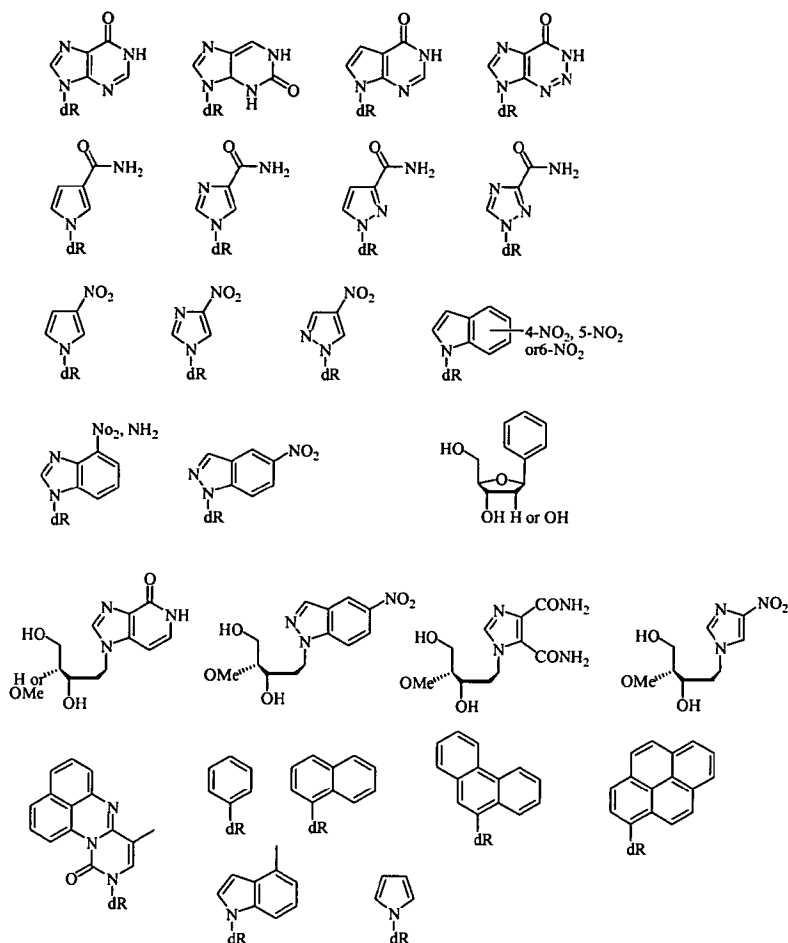
20 The enhanced binding affinity of the phenoxazine derivatives together with their uncompromised sequence specificity makes them valuable nucleobase analogs for the development of more potent antisense-based drugs. In fact, promising data have been derived from in vitro experiments demonstrating that heptanucleotides containing phenoxazine substitutions are capable to activate RNaseH, enhance cellular uptake and
25 exhibit an increased antisense activity [Lin, K.-Y.; Matteucci, M. J. Am. Chem. Soc. 1998, 120, 8531-8532]. The activity enhancement was even more pronounced in case of G-clamp, as a single substitution was shown to significantly improve the in vitro potency of a 20mer 2'-deoxyphosphorothioate oligonucleotides [Flanagan, W. M.; Wolf, J.J.; Olson, P.; Grant, D.; Lin, K.-Y.; Wagner, R. W.; Matteucci, M. Proc. Natl. Acad. Sci. USA, 1999,
30 96, 3513-3518]. Nevertheless, to optimize oligonucleotide design and to better understand the impact of these heterocyclic modifications on the biological activity, it is important to evaluate their effect on the nuclease stability of the oligomers.

Further modified polycyclic heterocyclic compounds useful as heterocyclic bases are disclosed in but not limited to, the above noted U.S. 3,687,808, as well as U.S.: 4,845,205; 5,130,302; 5,134,066; 5,175,273; 5,367,066; 5,432,272; 5,434,257; 5,457,187; 5,459,255; 5,484,908; 5,502,177; 5,525,711; 5,552,540; 5,587,469; 5,594,121, 5,596,091; 5,614,617; 5,645,985; 5,646,269; 5,750,692; 5,830,653; 5,763,588; 6,005,096; and 5,681,941, and United States Patent Application Serial number 09/996,292 filed November 28, 2001, certain of which are commonly owned with the instant application, and each of which is herein incorporated by reference.

ADDITIONAL MODIFIED NUCLEOBASES

10 The term “universal base” as used herein, refers to a monomer in a first sequence that can pair with a naturally occurring base, i.e A, C, G, T or U at a corresponding position in a second sequence of a duplex in which one or more of the following is true: (1) there is essentially no pairing between the two; or (2) the pairing between them occurs non-discriminantly with each of the naturally occurring bases and without significant
15 destabilization of the duplex.

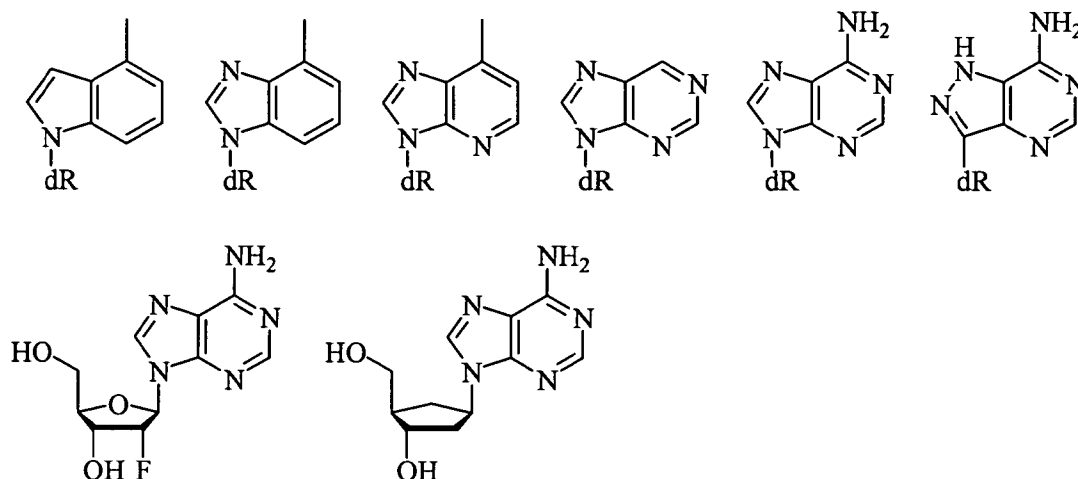
For examples of universal bases see Survey and summary: the applications of universal DNA base analogs. Loakes, D. *Nucleic Acids Research*, **2001**, 29, 12, 2437-2447.



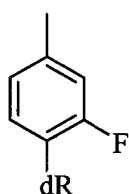
- The term “hydrophobic base” as used herein, refers to a monomer in a first sequence that can pair with a naturally occurring base, i.e A, C, G, T or U at a corresponding position in a second sequence of a duplex in which one or more of the following is true: (1) the
- 5 hydrophobic base acts as a non-polar close size and shape mimic (isostere) of one of the naturally occurring nucleosides; or (2) the hydrophobic base lacks all hydrogen bonding functionality on the Watson-Crick pairing edge.

For examples of adenine isosteres, see Probing the requirements for recognition and catalysis in Fpg and MutY with nonpolar adenine isosteres. Francis, AW, Helquist, SA,

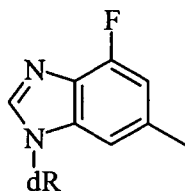
10 Kool, ET, David, SS. *J. Am. Chem. Soc.*, **2003**, *125*, 16235-16242 or Structure and base pairing properties of a replicable nonpolar isostere for deoxyadenosine. Guckian, KM, Morales, JC, Kool, ET. *J. Org. Chem.*, **1998**, *63*, 9652-9656.



- For an example of a cytosine isostere, see Hydrolysis of RNA/DNA hybrids containing nonpolar pyrimidine isosteres defines regions essential for HIV type polypurine tract selection. Rausch, JW, Qu, J, Yi-Brunozzi HY, Kool, ET, LeGrice, SFJ. *Proc. Natl. Acad. Sci.*, **2003**, *100*, 11279-11284.
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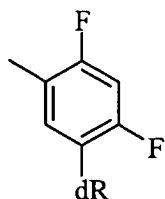


- For an example of a guanosine isostere, see A highly effective nonpolar isostere of deoxoguanosine: synthesis, structure, stacking and base pairing. O'Neil, BM, Ratto, JE, Good, KL, Tahmassebi, DC, Helquist, SA, Morales, JC, Kool, ET. *J. Org. Chem.*, **2002**, *67*, 5869-5875.
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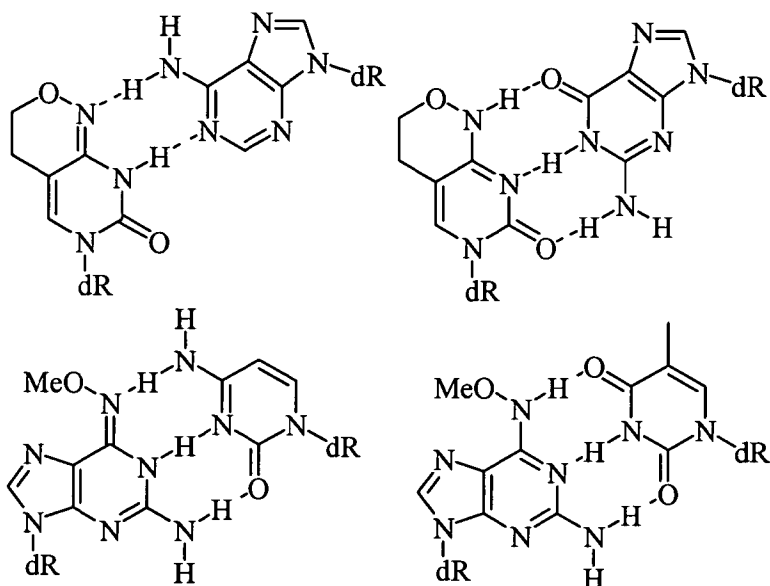


- For an example of a thymidine isostere, see A thymidine triphosphate shape analog lacking Watson-Crick pairing ability is replicated with high sequence selectivity. Moran, S, Ren, RX-F, Kool, ET. *Proc. Natl. Acad. Sci.*, **1997**, *94*, 10506-10511 or

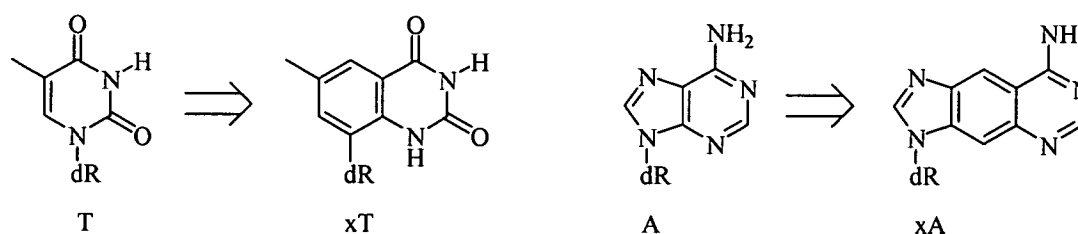
Difluorotoluene, a nonpolar isostere for thymidine, codes specifically and efficiently for adenine in DNA replication. *J. Am. Chem. Soc.* **1997**, *119*, 2056-2057.



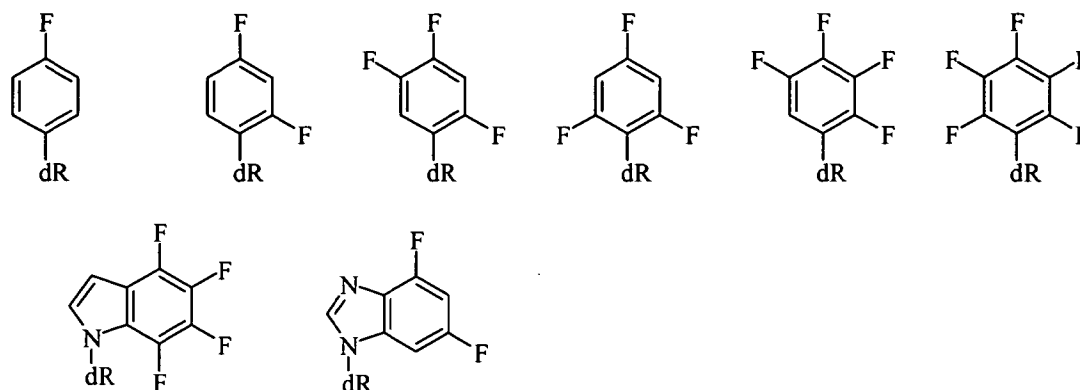
- 5 The term “promiscuous base” as used herein, refers to a monomer in a first sequence that can pair with a naturally occurring base, i.e. A, C, G, T or U at a corresponding position in a second sequence of a duplex in which the promiscuous base can pair non-discriminantly with more than one of the naturally occurring bases, i.e. A, C, G, T, U, but not with all four of them. For an example, see Polymerase recognition of synthetic
- 10 oligodeoxyribonucleotides incorporating degenerate pyrimidine and purine bases. Hill, F.; Loakes, D.; Brown, D. M. *Proc. Natl. Acad. Sci.*, **1998**, *95*, 4258-4263.



The term “size expanded base” as used herein, refers to analogs of naturally occurring nucleobases that are larger in size and retain their Watson-Crick pairing ability. For examples see A four-base paired genetic helix with expanded size. Liu, B, Gao, J, Lynch, SR, Saito, D, Maynard, L, Kool, ET., *Science*, **2003**, *302*, 868-871 and Toward a new
5 genetic system with expanded dimension: size expanded analogues of deoxyadenosine and thymidine. Liu, H, Goa, J, Maynard, Y, Saito, D, Kool, ET, *J. Am. Chem. Soc.* **2004**, *126*, 1102-1109.



10 The term fluorinated nucleobase as used herein, refers to a nucleobase or nucleobase analog, wherein one or more of the aromatic ring substituents is a fluorine atom. It may be possible that all of the ring substituents are fluorine atoms. For examples of fluorinated nucleobases see fluorinated DNA bases as probes of electrostatic effects in DNA base stacking. Lai, JS, QU, J, Kool, ET, *Angew. Chem. Int. Ed.*, **2003**, *42*, 5973-
15 5977 and Selective pairing of polyfluorinated DNA bases, Lai, JS, Kool, ET, *J. Am. Chem. Soc.*, **2004**, *126*, 3040-3041 and The effect of universal fluorinated nucleobases on the catalytic activity of ribozymes, Klopffer, AE, Engels, JW, *Nucleosides, Nucleotides & Nucleic Acids*, **2003**, *22*, 1347-1350 and Synthesis of 2' aminoalkyl-substituted fluorinated nucleobases and their influence on the kinetic properties of hammerhead ribozymes,
20 Klopffer, AE, Engels, JW, *ChemBioChem.*, **2003**, *5*, 707-716



Conjugates

Oligomeric compounds used in the compositions of the present invention can also
5 be modified to have one or more moieties or conjugates which enhance the activity,
cellular distribution or cellular uptake of the resulting oligomeric compounds. In one
embodiment such modified oligomeric compounds are prepared by covalently attaching
conjugate groups to functional groups such as hydroxyl or amino groups. Conjugate
groups of the invention include intercalators, reporter molecules, polyamines, polyamides,
10 polyethylene glycols, polyethers, groups that enhance the pharmacodynamic properties of
oligomers, and groups that enhance the pharmacokinetic properties of oligomers. Typical
conjugates groups include cholesterol, lipids, phospholipids, biotin, phenazine, folate,
phenanthridine, anthraquinone, acridine, fluoresceins, rhodamines, coumarins, and dyes.
Groups that enhance the pharmacodynamic properties, in the context of this invention,
15 include groups that improve oligomer uptake, enhance oligomer resistance to degradation,
and/or strengthen sequence-specific hybridization with RNA. Groups that enhance the
pharmacokinetic properties, in the context of this invention, include groups that improve
oligomer uptake, distribution, metabolism or excretion. Representative conjugate groups
are disclosed in International Patent Application PCT/US92/09196, filed October 23, 1992
20 the entire disclosure of which is incorporated herein by reference. Conjugate moieties
include but are not limited to lipid moieties such as a cholesterol moiety (Letsinger et al.,
Proc. Natl. Acad. Sci. USA, **1989**, *86*, 6553-6556), cholic acid (Manoharan et al., *Bioorg.*
Med. Chem. Let., **1994**, *4*, 1053-1060), a thioether, e.g., hexyl-S-tritylthiol (Manoharan et
al., *Ann. N.Y. Acad. Sci.*, **1992**, *660*, 306-309; Manoharan et al., *Bioorg. Med. Chem. Let.*,

1993, 3, 2765-2770), a thiocholesterol (Oberhauser et al., *Nucl. Acids Res.*, 1992, 20, 533-538), an aliphatic chain, e.g., dodecandiol or undecyl residues (Saison-Behmoaras et al., *EMBO J.*, 1991, 10, 1111-1118; Kabanov et al., *FEBS Lett.*, 1990, 259, 327-330; Svinarchuk et al., *Biochimie*, 1993, 75, 49-54), a phospholipid, e.g., di-hexadecyl-rac-glycerol or triethylammonium 1,2-di-O-hexadecyl-rac-glycero-3-H-phosphonate
5 (Manoharan et al., *Tetrahedron Lett.*, 1995, 36, 3651-3654; Shea et al., *Nucl. Acids Res.*, 1990, 18, 3777-3783), a polyamine or a polyethylene glycol chain (Manoharan et al., *Nucleosides & Nucleotides*, 1995, 14, 969-973), or adamantane acetic acid (Manoharan et al., *Tetrahedron Lett.*, 1995, 36, 3651-3654), a palmityl moiety (Mishra et al., *Biochim.*
10 *Biophys. Acta*, 1995, 1264, 229-237), or an octadecylamine or hexylamino-carbonyl-oxycholesterol moiety (Crooke et al., *J. Pharmacol. Exp. Ther.*, 1996, 277, 923-937).

The oligomeric compounds of the invention may also be conjugated to active drug substances, for example, aspirin, warfarin, phenylbutazone, ibuprofen, suprofen, fenbufen, ketoprofen, (*S*)-(+)-pranoprofen, carprofen, dansylsarcosine, 2,3,5-triodobenzoic acid,
15 flufenamic acid, folinic acid, a benzothiadiazide, chlorothiazide, a diazepine, indomethicin, a barbiturate, a cephalosporin, a sulfa drug, an antidiabetic, an antibacterial or an antibiotic. Oligonucleotide-drug conjugates and their preparation are described in United States Patent Application 09/334,130 (filed June 15, 1999) which is incorporated herein by reference in its entirety.

20 Representative United States patents that teach the preparation of such oligonucleotide conjugates include, but are not limited to, U.S.: 4,828,979; 4,948,882; 5,218,105; 5,525,465; 5,541,313; 5,545,730; 5,552,538; 5,578,717; 5,580,731; 5,580,731; 5,591,584; 5,109,124; 5,118,802; 5,138,045; 5,414,077; 5,486,603; 5,512,439; 5,578,718; 5,608,046; 4,587,044; 4,605,735; 4,667,025; 4,762,779; 4,789,737; 4,824,941; 4,835,263;
25 4,876,335; 4,904,582; 4,958,013; 5,082,830; 5,112,963; 5,214,136; 5,082,830; 5,112,963; 5,214,136; 5,245,022; 5,254,469; 5,258,506; 5,262,536; 5,272,250; 5,292,873; 5,317,098; 5,371,241; 5,391,723; 5,416,203; 5,451,463; 5,510,475; 5,512,667; 5,514,785; 5,565,552; 5,567,810; 5,574,142; 5,585,481; 5,587,371; 5,595,726; 5,597,696; 5,599,923; 5,599,928 and 5,688,941, certain of which are commonly owned with the instant application, and
30 each of which is herein incorporated by reference.

3'-Endo Modifications

In one aspect of the present invention oligomeric compounds include nucleosides synthetically modified to induce a 3'-endo sugar conformation. A nucleoside can incorporate synthetic modifications of the heterocyclic base moiety, the sugar moiety or both to induce a desired 3'-endo sugar conformation. These modified nucleosides are used to mimic RNA like nucleosides so that particular properties of an oligomeric compound can be enhanced while maintaining the desirable 3'-endo conformational geometry. There is an apparent preference for an RNA type duplex (A form helix, predominantly 3'-endo) as a requirement of RNA interference which is supported in part by the fact that duplexes composed of 2'-deoxy-2'-F-nucleosides appear efficient in triggering RNAi response in the *C. elegans* system. Properties that are enhanced by using more stable 3'-endo nucleosides include but aren't limited to modulation of pharmacokinetic properties through modification of protein binding, protein off-rate, absorption and clearance; modulation of nuclease stability as well as chemical stability; modulation of the binding affinity and specificity of the oligomer (affinity and specificity for enzymes as well as for complementary sequences); and increasing efficacy of RNA cleavage. The present invention provides oligomeric compounds having one or more nucleosides modified in such a way as to favor a C3'-endo type conformation.

Scheme 1



C2'-endo/Southern

C3'-endo/Northern

Nucleoside conformation is influenced by various factors including substitution at the 2', 3' or 4'-positions of the pentofuranosyl sugar. Electronegative substituents generally prefer the axial positions, while sterically demanding substituents generally prefer the equatorial positions (Principles of Nucleic Acid Structure, Wolfgang Sanger, 1984, Springer-Verlag.) Modification of the 2' position to favor the 3'-endo conformation can be achieved while maintaining the 2'-OH as a recognition element, as illustrated in Figure 2, below (Gallo et al., Tetrahedron (2001), 57, 5707-5713. Harry-O'kuru et al., J. Org.

Chem., (1997), 62(6), 1754-1759 and Tang et al., J. Org. Chem. (1999), 64, 747-754.)

Alternatively, preference for the 3'-endo conformation can be achieved by deletion of the 2'-OH as exemplified by 2'-deoxy-2'-F-nucleosides (Kawasaki et al., J. Med. Chem. (1993), 36, 831-841), which adopts the 3'-endo conformation positioning the electronegative fluorine atom in the axial position. Other modifications of the ribose ring, for example substitution at the 4'-position to give 4'-F modified nucleosides (Guillerm et al., Bioorganic and Medicinal Chemistry Letters (1995), 5, 1455-1460 and Owen et al., J. Org. Chem. (1976), 41, 3010-3017), or for example modification to yield methanocarba nucleoside analogs (Jacobson et al., J. Med. Chem. Lett. (2000), 43, 2196-2203 and Lee et al., Bioorganic and Medicinal Chemistry Letters (2001), 11, 1333-1337) also induce preference for the 3'-endo conformation. Some modifications actually lock the conformational geometry by formation of a bicyclic sugar moiety e.g. locked nucleic acid (LNA, Singh et al, Chem. Commun. (1998), 4, 455-456), and ethylene bridged nucleic acids (ENA, Morita et al, Bioorganic & Medicinal Chemistry Letters (2002), 12, 73-76.)

Examples of modified nucleosides amenable to the present invention are shown below in Table I. These examples are meant to be representative and not exhaustive.

for use in one or more of the oligomeric compounds of the present invention. The synthesis of numerous of the modified nucleosides amenable to the present invention are known in the art (see for example, Chemistry of Nucleosides and Nucleotides Vol 1-3, ed. Leroy B. Townsend, 1988, Plenum press., and the examples section below.) Nucleosides
5 known to be inhibitors/substrates for RNA dependent RNA polymerases (for example HCV NS5B

In one aspect, the present invention is directed to oligomeric compounds that are prepared having enhanced properties compared to native RNA against nucleic acid targets. A target is identified and an oligomeric compound is selected having an effective length
10 and sequence that is complementary to a portion of the target sequence. Each nucleoside of the selected sequence is scrutinized for possible enhancing modifications. A preferred modification would be the replacement of one or more RNA nucleosides with nucleosides that have the same 3'-endo conformational geometry. Such modifications can enhance chemical and nuclease stability relative to native RNA while at the same time being much
15 cheaper and easier to synthesize and/or incorporate into an oligomeric compound. The selected sequence can be further divided into regions and the nucleosides of each region evaluated for enhancing modifications that can be the result of a chimeric configuration. Consideration is also given to the termini (e.g. 5' and 3'-termini) as there are often advantageous modifications that can be made to one or more of the terminal monomeric
20 subunits. In one aspect of the invention, desired properties and or activity of oligomeric compounds are enhanced by the inclusion of a 5'-phosphate or modified phosphate moiety.

The terms used to describe the conformational geometry of homoduplex nucleic acids are "A Form" for RNA and "B Form" for DNA. The respective conformational geometry for RNA and DNA duplexes was determined from X-ray diffraction analysis of
25 nucleic acid fibers (Arnott and Hukins, *Biochem. Biophys. Res. Comm.*, 1970, 47, 1504.) In general, RNA:RNA duplexes are more stable and have higher melting temperatures (T_m's) than DNA:DNA duplexes (Sanger et al., Principles of Nucleic Acid Structure, 1984, Springer-Verlag; New York, NY.; Lesnik et al., Biochemistry, 1995, 34, 10807-10815; Conte et al., Nucleic Acids Res., 1997, 25, 2627-2634). The increased stability of
30 RNA has been attributed to several structural features, most notably the improved base stacking interactions that result from an A-form geometry (Searle et al., Nucleic Acids Res., 1993, 21, 2051-2056). The presence of the 2' hydroxyl in RNA biases the sugar

toward a C3' endo pucker, i.e., also designated as Northern pucker, which causes the duplex to favor the A-form geometry. In addition, the 2' hydroxyl groups of RNA can form a network of water mediated hydrogen bonds that help stabilize the RNA duplex (Egli et al., *Biochemistry*, 1996, 35, 8489-8494). On the other hand, deoxy nucleic acids
5 prefer a C2' endo sugar pucker, i.e., also known as Southern pucker, which is thought to impart a less stable B-form geometry (Sanger, W. (1984) *Principles of Nucleic Acid Structure*, Springer-Verlag, New York, NY). As used herein, B-form geometry is inclusive of both C2'-endo pucker and O4'-endo pucker. This is consistent with Berger, *et al.*, *Nucleic Acids Research*, 1998, 26, 2473-2480, who pointed out that in considering the
10 furanose conformations which give rise to B-form duplexes consideration should also be given to a O4'-endo pucker contribution.

DNA:RNA hybrid duplexes, however, are usually less stable than pure RNA:RNA duplexes, and depending on their sequence may be either more or less stable than DNA:DNA duplexes (Searle *et al.*, *Nucleic Acids Res.*, 1993, 21, 2051-2056). The
15 structure of a hybrid duplex is intermediate between A- and B-form geometries, which may result in poor stacking interactions (Lane *et al.*, *Eur. J. Biochem.*, 1993, 215, 297-306; Fedoroff *et al.*, *J. Mol. Biol.*, 1993, 233, 509-523; Gonzalez *et al.*, *Biochemistry*, 1995, 34, 4969-4982; Horton *et al.*, *J. Mol. Biol.*, 1996, 264, 521-533). The stability of the duplex formed between a target RNA and a synthetic sequence is central to therapies such
20 as but not limited to antisense and RNA interference as these mechanisms require the binding of a synthetic strand of oligomeric compound to an RNA target strand. In the case of antisense, effective inhibition of the mRNA requires that the antisense DNA have a very high binding affinity with the mRNA. Otherwise the desired interaction between the synthetic strand and target mRNA strand will occur infrequently, resulting in decreased
25 efficacy.

One routinely used method of modifying the sugar pucker is the substitution of the sugar at the 2'-position with a substituent group that influences the sugar geometry. The influence on ring conformation is dependant on the nature of the substituent at the 2'-position. A number of different substituents have been studied to determine their sugar
30 pucker effect. For example, 2'-halogens have been studied showing that the 2'-fluoro derivative exhibits the largest population (65%) of the C3'-endo form, and the 2'-iodo exhibits the lowest population (7%). The populations of adenosine (2'-OH) versus deoxy-

adenosine (2'-H) are 36% and 19%, respectively. Furthermore, the effect of the 2'-fluoro group of adenosine dimers (2'-deoxy-2'-fluoroadenosine - 2'-deoxy-2'-fluoro-adenosine) is further correlated to the stabilization of the stacked conformation.

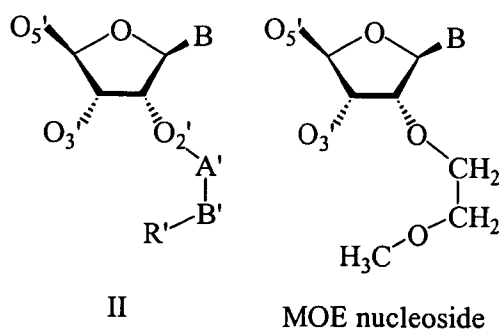
As expected, the relative duplex stability can be enhanced by replacement of 2'-OH groups with 2'-F groups thereby increasing the C3'-endo population. It is assumed that the highly polar nature of the 2'-F bond and the extreme preference for C3'-endo puckering may stabilize the stacked conformation in an A-form duplex. Data from UV hypochromicity, circular dichroism, and ¹H NMR also indicate that the degree of stacking decreases as the electronegativity of the halo substituent decreases. Furthermore, steric bulk at the 2'-position of the sugar moiety is better accommodated in an A-form duplex than a B-form duplex. Thus, a 2'-substituent on the 3'-terminus of a dinucleoside monophosphate is thought to exert a number of effects on the stacking conformation: steric repulsion, furanose puckering preference, electrostatic repulsion, hydrophobic attraction, and hydrogen bonding capabilities. These substituent effects are thought to be determined by the molecular size, electronegativity, and hydrophobicity of the substituent. Melting temperatures of complementary strands is also increased with the 2'-substituted adenosine diphosphates. It is not clear whether the 3'-endo preference of the conformation or the presence of the substituent is responsible for the increased binding. However, greater overlap of adjacent bases (stacking) can be achieved with the 3'-endo conformation.

One synthetic 2'-modification that imparts increased nuclease resistance and a very high binding affinity to nucleotides is the 2-methoxyethoxy (2'-MOE, 2'-OCH₂CH₂OCH₃) side chain (Baker *et al.*, *J. Biol. Chem.*, **1997**, 272, 11944-12000). One of the immediate advantages of the 2'-MOE substitution is the improvement in binding affinity, which is greater than many similar 2' modifications such as *O*-methyl, *O*-propyl, and *O*-aminopropyl. Oligonucleotides having the 2'-*O*-methoxyethyl substituent also have been shown to be antisense inhibitors of gene expression with promising features for *in vivo* use (Martin, P., *Helv. Chim. Acta*, **1995**, 78, 486-504; Altmann *et al.*, *Chimia*, **1996**, 50, 168-176; Altmann *et al.*, *Biochem. Soc. Trans.*, **1996**, 24, 630-637; and Altmann *et al.*, *Nucleosides Nucleotides*, **1997**, 16, 917-926). Relative to DNA, the oligonucleotides having the 2'-MOE modification displayed improved RNA affinity and higher nuclease resistance. Chimeric oligonucleotides having 2'-MOE substituents in the wing nucleosides

and an internal region of deoxy-phosphorothioate nucleotides (also termed a gapped oligonucleotide or gapmer) have shown effective reduction in the growth of tumors in animal models at low doses. 2'-MOE substituted oligonucleotides have also shown outstanding promise as antisense agents in several disease states. One such MOE substituted oligonucleotide is presently being investigated in clinical trials for the treatment of CMV retinitis.

To better understand the higher RNA affinity of 2'-*O*-methoxyethyl substituted RNA and to examine the conformational properties of the 2'-*O*-methoxyethyl substituent, two dodecamer oligonucleotides were synthesized having SEQ ID NO: 10 (CGC GAA UUC GCG) and SEQ ID NO: 11 (GCG CUU AAG CGC). These self-complementary strands have every 2'-position modified with a 2'-*O*-methoxyethyl. The duplex was crystallized at a resolution of 1.7 Ångstrom and the crystal structure was determined. The conditions used for the crystallization were 2 mM oligonucleotide, 50 mM Na HEPES pH 6.2-7.5, 10.50 mM MgCl₂, 15% PEG 400. The crystal data showed: space group C2, cell constants $a=41.2$ Å, $b=34.4$ Å, $c=46.6$ Å, $\beta=92.4^\circ$. The resolution was 1.7 Å at -170°C. The current R -factor was 20% (R_{free} 26%).

This crystal structure is believed to be the first crystal structure of a fully modified RNA oligonucleotide analogue. The duplex adopts an overall A-form conformation and all modified sugars display C3'-*endo* pucker. In most of the 2'-*O*-substituents, the torsion angle around the A'-B' bond, as depicted in Structure II below, of the ethylene glycol linker has a *gauche* conformation. For 2'-*O*-MOE, A' and B' of Structure II below are methylene moieties of the ethyl portion of the MOE and R' is the methoxy portion.



In the crystal, the 2'-O-MOE RNA duplex adopts a general orientation such that the crystallographic 2-fold rotation axis does not coincide with the molecular 2-fold rotation axis. The duplex adopts the expected A-type geometry and all of the 24 2'-O-MOE substituents were visible in the electron density maps at full resolution. The electron
5 density maps as well as the temperature factors of substituent atoms indicate flexibility of the 2'-O-MOE substituent in some cases.

Most of the 2'-O-MOE substituents display a *gauche* conformation around the C-C bond of the ethyl linker. However, in two cases, a *trans* conformation around the C-C bond is observed. The lattice interactions in the crystal include packing of duplexes
10 against each other via their minor grooves. Therefore, for some residues, the conformation of the 2'-O-substituent is affected by contacts to an adjacent duplex. In general, variations in the conformation of the substituents (*e.g.* g^+ or g^- around the C-C bonds) create a range of interactions between substituents, both inter-strand, across the minor groove, and intra-strand. At one location, atoms of substituents from two residues
15 are in van der Waals contact across the minor groove. Similarly, a close contact occurs between atoms of substituents from two adjacent intra-strand residues.

Previously determined crystal structures of A-DNA duplexes were for those that incorporated isolated 2'-O-methyl T residues. In the crystal structure noted above for the 2'-O-MOE substituents, a conserved hydration pattern has been observed for the 2'-O-
20 MOE residues. A single water molecule is seen located between O2', O3' and the methoxy oxygen atom of the substituent, forming contacts to all three of between 2.9 and 3.4 Å. In addition, oxygen atoms of substituents are involved in several other hydrogen bonding contacts. For example, the methoxy oxygen atom of a particular 2'-O-substituent forms a hydrogen bond to N3 of an adenosine from the opposite strand via a bridging water
25 molecule.

In several cases a water molecule is trapped between the oxygen atoms O2', O3' and OC' of modified nucleosides. 2'-O-MOE substituents with *trans* conformation around the C-C bond of the ethylene glycol linker are associated with close contacts between OC' and N2 of a guanosine from the opposite strand, and, water-mediated,
30 between OC' and N3(G). When combined with the available thermodynamic data for duplexes containing 2'-O-MOE modified strands, this crystal structure allows for further detailed structure-stability analysis of other modifications.

In extending the crystallographic structure studies, molecular modeling experiments were performed to study further enhanced binding affinity of oligonucleotides having 2'-O-modifications. The computer simulations were conducted on compounds of SEQ ID NO: 10, above, having 2'-O-modifications located at each of the nucleosides of the oligonucleotide. The simulations were performed with the oligonucleotide in aqueous solution using the AMBER force field method (Cornell *et al.*, *J. Am. Chem. Soc.*, **1995**, *117*, 5179-5197)(modeling software package from UCSF, San Francisco, CA). The calculations were performed on an Indigo2 SGI machine (Silicon Graphics, Mountain View, CA).

Further 2'-O-modifications that will have a 3'-endo sugar influence include those having a ring structure that incorporates a two atom portion corresponding to the A' and B' atoms of Structure II. The ring structure is attached at the 2' position of a sugar moiety of one or more nucleosides that are incorporated into an oligonucleotide. The 2'-oxygen of the nucleoside links to a carbon atom corresponding to the A' atom of Structure II. These ring structures can be aliphatic, unsaturated aliphatic, aromatic or heterocyclic. A further atom of the ring (corresponding to the B' atom of Structure II), bears a further oxygen atom, or a sulfur or nitrogen atom. This oxygen, sulfur or nitrogen atom is bonded to one or more hydrogen atoms, alkyl moieties, or haloalkyl moieties, or is part of a further chemical moiety such as a ureido, carbamate, amide or amidine moiety. The remainder of the ring structure restricts rotation about the bond joining these two ring atoms. This assists in positioning the "further oxygen, sulfur or nitrogen atom" (part of the R position as described above) such that the further atom can be located in close proximity to the 3'-oxygen atom (O3') of the nucleoside.

Another preferred 2'-sugar substituent group that gives a 3'-endo sugar conformational geometry is the 2'-OMe group. 2'-Substitution of guanosine, cytidine, and uridine dinucleoside phosphates with the 2'-OMe group showed enhanced stacking effects with respect to the corresponding native (2'-OH) species leading to the conclusion that the sugar is adopting a C3'-endo conformation. In this case, it is believed that the hydrophobic attractive forces of the methyl group tend to overcome the destabilizing effects of its steric bulk.

The ability of oligonucleotides to bind to their complementary target strands is compared by determining the melting temperature (T_m) of the hybridization complex of

the oligonucleotide and its complementary strand. The melting temperature (T_m), a characteristic physical property of double helices, denotes the temperature (in degrees centigrade) at which 50% helical (hybridized) versus coil (unhybridized) forms are present. T_m is measured by using the UV spectrum to determine the formation and
5 breakdown (melting) of the hybridization complex. Base stacking, which occurs during hybridization, is accompanied by a reduction in UV absorption (hypochromicity). Consequently, a reduction in UV absorption indicates a higher T_m . The higher the T_m , the greater the strength of the bonds between the strands.

Freier and Altmann, *Nucleic Acids Research*, (1997) 25:4429-4443, have
10 previously published a study on the influence of structural modifications of oligonucleotides on the stability of their duplexes with target RNA. In this study, the authors reviewed a series of oligonucleotides containing more than 200 different modifications that had been synthesized and assessed for their hybridization affinity and T_m . Sugar modifications studied included substitutions on the 2'-position of the sugar, 3'-
15 substitution, replacement of the 4'-oxygen, the use of bicyclic sugars, and four member ring replacements. Several nucleobase modifications were also studied including substitutions at the 5, or 6 position of thymine, modifications of pyrimidine heterocycle and modifications of the purine heterocycle. Modified internucleoside linkages were also studied including neutral, phosphorus and non-phosphorus containing internucleoside
20 linkages.

Increasing the percentage of C3'-endo sugars in a modified oligonucleotide targeted to an RNA target strand should preorganize this strand for binding to RNA. Of the several sugar modifications that have been reported and studied in the literature, the incorporation of electronegative substituents such as 2'-fluoro or 2'-alkoxy shift the sugar
25 conformation towards the 3' endo (northern) pucker conformation. This preorganizes an oligonucleotide that incorporates such modifications to have an A-form conformational geometry. This A-form conformation results in increased binding affinity of the oligonucleotide to a target RNA strand.

Molecular modeling experiments were performed to study further enhanced
30 binding affinity of oligonucleotides having 2'-O-modifications. Computer simulations were conducted on compounds having SEQ ID NO: 10, r(CGC GAA UUC GCG), having 2'-O-modifications of the invention located at each of the nucleoside of the

oligonucleotide. The simulations were performed with the oligonucleotide in aqueous solution using the AMBER force field method (Cornell *et al.*, *J. Am. Chem. Soc.*, 1995, 117, 5179-5197)(modeling software package from UCSF, San Francisco, CA). The calculations were performed on an Indigo2 SGI machine (Silicon Graphics, Mountain View, CA).

In addition, for 2'-substituents containing an ethylene glycol motif, a *gauche* interaction between the oxygen atoms around the O-C-C-O torsion of the side chain may have a stabilizing effect on the duplex (Freier *ibid.*). Such *gauche* interactions have been observed experimentally for a number of years (Wolfe *et al.*, *Acc. Chem. Res.*, 1972, 5, 102; Abe *et al.*, *J. Am. Chem. Soc.*, 1976, 98, 468). This *gauche* effect may result in a configuration of the side chain that is favorable for duplex formation. The exact nature of this stabilizing configuration has not yet been explained. While we do not want to be bound by theory, it may be that holding the O-C-C-O torsion in a single *gauche* configuration, rather than a more random distribution seen in an alkyl side chain, provides an entropic advantage for duplex formation.

Representative 2'-substituent groups amenable to the present invention that give A-form conformational properties (3'-endo) to the resultant duplexes include 2'-O-alkyl, 2'-O-substituted alkyl and 2'-fluoro substituent groups. Preferred for the substituent groups are various alkyl and aryl ethers and thioethers, amines and monoalkyl and dialkyl substituted amines. It is further intended that multiple modifications can be made to one or more of the oligomeric compounds of the invention at multiple sites of one or more monomeric subunits (nucleosides are preferred) and or internucleoside linkages to enhance properties such as but not limited to activity in a selected application. Tables I through VII list nucleoside and internucleotide linkage modifications/replacements that have been shown to give a positive ΔT_m per modification when the modification/replacement was made to a DNA strand that was hybridized to an RNA complement.

Table I

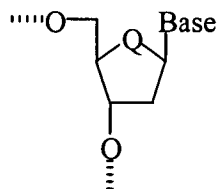
Modified DNA strand having 2'-substituent groups that gave an overall increase in T_m against an RNA complement:

| | |
|----|--|
| 5 | <u>Positive ΔT_m/mod</u> |
| | 2'-substituents |
| | 2'-OH |
| | 2'-O-C ₁ -C ₄ alkyl |
| | 2'-O-(CH ₂) ₂ CH ₃ |
| | 2'-O-CH ₂ CH=CH ₂ |
| 10 | 2'-F |
| | 2'-O-(CH ₂) ₂ -O-CH ₃ |
| | 2'-[O-(CH ₂) ₂] ₂ -O-CH ₃ |
| | 2'-[O-(CH ₂) ₂] ₃ -O-CH ₃ |
| | 2'-[O-(CH ₂) ₂] ₄ -O-CH ₃ |
| 15 | 2'-[O-(CH ₂) ₂] ₃ -O-(CH ₂) ₈ CH ₃ |
| | 2'-O-(CH ₂) ₂ CF ₃ |
| | 2'-O-(CH ₂) ₂ OH |
| | 2'-O-(CH ₂) ₂ F |
| | 2'-O-CH ₂ CH(CH ₃)F |
| 20 | 2'-O-CH ₂ CH(CH ₂ OH)OH |
| | 2'-O-CH ₂ CH(CH ₂ OCH ₃)OCH ₃ |
| | 2'-O-CH ₂ CH(CH ₃)OCH ₃ |
| | 2'-O-CH ₂ -C ₁₄ H ₇ O ₂ (-C ₁₄ H ₇ O ₂ = Anthraquinone) |
| | 2'-O-(CH ₂) ₃ -NH ₂ * |
| 25 | 2'-O-(CH ₂) ₄ -NH ₂ * |

* These modifications can increase the T_m of oligonucleotides but can also decrease the T_m depending on positioning and number (motif dependant).

Table II

Modified DNA strand having modified sugar ring (see structure x) that gave an overall increase in T_m against an RNA complement:



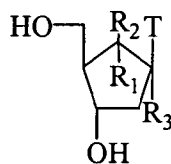
5 Positive $\epsilon T_m/\text{mod}$

Q -S-
 -CH₂-

Note: In general ring oxygen substitution with sulfur or methylene had only a minor effect on T_m for the specific motifs studied. Substitution at the 2'-position with groups shown to stabilize the duplex were destabilizing when CH₂ replaced the ring O. This is thought to be due to the necessary gauche interaction between the ring O with particular 2'-substituents (for example -O-CH₃ and -(O-CH₂CH₂)₃-O-CH₃).

Table III

15 Modified DNA strand having modified sugar ring that give an overall increase in T_m against an RNA complement:



Positive $\epsilon T_m/\text{mod}$

-C(H)R₁ effects OH
20 (R₂, R₃ both = H) CH₃*
 CH₂OH*
 OCH₃*

* These modifications can increase the T_m of oligonucleotides but can also decrease the T_m depending on positioning and number (motif dependant).

Table IV

Modified DNA strand having bicyclic substitute sugar modifications that give an overall increase in T_m against an RNA complement:

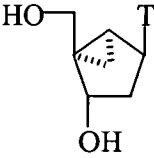
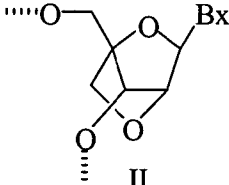
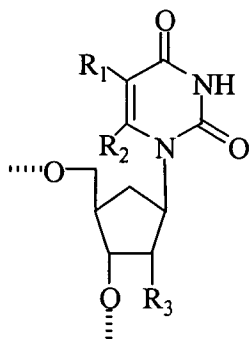
| | | |
|---|---|---|
| 5 | Formula | <u>Positive ϵT_m/mod</u> |
| | I | + |
| | II | + |
| |  | |
| |  | |

Table V

Modified DNA strand having modified heterocyclic base moieties that give an overall increase in T_m against an RNA complement:

| | | |
|----|------------------------------------|---|
| 5 | <u>Modification/Formula</u> | <u>Positive \in T_m/mod</u> |
| | Heterocyclic base | 2-thioT |
| | modifications | 2'-O-methylpseudoU |
| | | 7-halo-7-deaza purines |
| | | 7-propyne-7-deaza purines |
| 10 | | 2-aminoA(2,6-diaminopurine) |

| | | |
|--|------------------------------------|---|
| | <u>Modification/Formula</u> | <u>Positive \in T_m/mod</u> |
|--|------------------------------------|---|



(R₂, R₃=H), R₁=

15

Br

C/C-CH₃(CH₂)₃NH₂CH₃

Motiffs-disubstitution

R₁= C/C-CH₃, R₂=H, R₃=

F

20

R₁= C/C-CH₃, R₂=H

R₃= O-(CH₂)₂-O-CH₃

R₁= O-CH₃, R₂=H,

R₃= O-(CH₂)₂-O-CH₃*

* This modification can increase the T_m of oligonucleotides but can also decrease the T_m depending on positioning and number (motiff dependant).

Substitution at R₁ can be stabilizing, substitution at R₂ is generally greatly destabilizing (unable to form anti conformation), motifs with stabilizing 5 and 2'-substituent groups are generally additive e.g. increase stability.

5 Substitution of the O4 and O2 positions of 2'-O-methyl uridine was greatly duplex destabilizing as these modifications remove hydrogen binding sites that would be an expected result. 6-Aza T also showed extreme destabilization as this substitution reduces the pK_a and shifts the nucleoside toward the enol tautomer resulting in reduced hydrogen bonding.

Table VI

**DNA strand having at least one modified phosphorus containing
internucleoside linkage and the effect on the T_m against an RNA complement:**

| | | |
|----|----------------------------------|---|
| 5 | ∈T _m /mod + | ∈T _m /mod - |
| | | phosphorothioate ¹ |
| | | phosphoramidate ¹ |
| | | methyl phosphonates ¹ |
| | | (¹ one of the non-bridging oxygen atoms |
| 10 | | replaced with S, N(H)R or -CH ₃) |
| | phosphoramidate (the 3'-bridging | |
| | atom replaced with an N(H)R | |
| | group, stabilization effect | |
| | enhanced when also have 2'-F) | |
| 15 | | |

Table VII

DNA strand having at least one non-phosphorus containing internucleoside linkage and the effect on the T_m against an RNA complement:

| | |
|----|---|
| 5 | Positive $\Delta T_m/\text{mod}$ |
| | -CH ₂ C(=O)NHCH ₂ -* |
| | -CH ₂ C(=O)N(CH ₃)CH ₂ -* |
| | -CH ₂ C(=O)N(CH ₂ CH ₂ CH ₃)CH ₂ -* |
| | -CH ₂ C(=O)N(H)CH ₂ - (motiff with 5'-propyne on T's) |
| 10 | -CH ₂ N(H)C(=O)CH ₂ -* |
| | -CH ₂ N(CH ₃)OCH ₂ -* |
| | -CH ₂ N(CH ₃)N(CH ₃)CH ₂ -* |

* This modification can increase the T_m of oligonucleotides but can also decrease the T_m depending on positioning and number (motiff dependant).

Notes: In general carbon chain internucleotide linkages were destabilizing to duplex formation. This destabilization was not as severe when double and tripple bonds were utilized. The use of glycol and flexible ether linkages were also destabilizing.

Preferred ring structures of the invention for inclusion as a 2'-O modification include cyclohexyl, cyclopentyl and phenyl rings as well as heterocyclic rings having spacial footprints similar to cyclohexyl, cyclopentyl and phenyl rings. Particularly preferred 2'-O-substituent groups of the invention are listed below including an abbreviation for each:

| | |
|----|--|
| | 2'-O-(trans 2-methoxy cyclohexyl) -- 2'-O-(TMCHL) |
| 25 | 2'-O-(trans 2-methoxy cyclopentyl) -- 2'-O-(TMCPL) |
| | 2'-O-(trans 2-ureido cyclohexyl) -- 2'-O-(TUCHL) |
| | 2'-O-(trans 2-methoxyphenyl) -- 2'-O-(2MP) |

Structural details for duplexes incorporating such 2'-O-substituents were analyzed using the described AMBER force field program on the Indigo2 SGI machine. The simulated structure maintained a stable A-form geometry throughout the duration of the simulation. The presence of the 2' substitutions locked the sugars in the C3'-endo conformation.

The simulation for the TMCHL modification revealed that the 2'-O-(TMCHL) side chains have a direct interaction with water molecules solvating the duplex. The oxygen atoms in the 2'-O-(TMCHL) side chain are capable of forming a water-mediated interaction with the 3' oxygen of the phosphate backbone. The presence of the two oxygen
5 atoms in the 2'-O-(TMCHL) side chain gives rise to favorable gauche interactions. The barrier for rotation around the O-C-C-O torsion is made even larger by this novel modification. The preferential preorganization in an A-type geometry increases the binding affinity of the 2'-O-(TMCHL) to the target RNA. The locked side chain conformation in the 2'-O-(TMCHL) group created a more favorable pocket for binding
10 water molecules. The presence of these water molecules played a key role in holding the side chains in the preferable gauche conformation. While not wishing to be bound by theory, the bulk of the substituent, the diequatorial orientation of the substituents in the cyclohexane ring, the water of hydration and the potential for trapping of metal ions in the conformation generated will additionally contribute to improved binding affinity and
15 nuclease resistance of oligonucleotides incorporating nucleosides having this 2'-O-modification.

As described for the TMCHL modification above, identical computer simulations of the 2'-O-(TMCPL), the 2'-O-(2MP) and 2'-O-(TUCHL) modified oligonucleotides in aqueous solution also illustrate that stable A-form geometry will be maintained throughout
20 the duration of the simulation. The presence of the 2' substitution will lock the sugars in the C3'-endo conformation and the side chains will have direct interaction with water molecules solvating the duplex. The oxygen atoms in the respective side chains are capable of forming a water-mediated interaction with the 3' oxygen of the phosphate backbone. The presence of the two oxygen atoms in the respective side chains give rise to
25 the favorable gauche interactions. The barrier for rotation around the respective O-C-C-O torsions will be made even larger by respective modification. The preferential preorganization in A-type geometry will increase the binding affinity of the respective 2'-O-modified oligonucleotides to the target RNA. The locked side chain conformation in the respective modifications will create a more favorable pocket for binding water molecules.
30 The presence of these water molecules plays a key role in holding the side chains in the preferable gauche conformation. The bulk of the substituent, the diequatorial orientation of the substituents in their respective rings, the water of hydration and the potential

trapping of metal ions in the conformation generated will all contribute to improved binding affinity and nuclease resistance of oligonucleotides incorporating nucleosides having these respective 2'-O-modification.

Ribose conformations in C2'-modified nucleosides containing S-methyl groups were examined. To understand the influence of 2'-O-methyl and 2'-S-methyl groups on the conformation of nucleosides, we evaluated the relative energies of the 2'-O- and 2'-S-methylguanosine, along with normal deoxyguanosine and riboguanosine, starting from both C2'-endo and C3'-endo conformations using *ab initio* quantum mechanical calculations. All the structures were fully optimized at HF/6-31G* level and single point energies with electron-correlation were obtained at the MP2/6-31G*//HF/6-31G* level. As shown in Table 1, the C2'-endo conformation of deoxyguanosine is estimated to be 0.6 kcal/mol more stable than the C3'-endo conformation in the gas-phase. The conformational preference of the C2'-endo over the C3'-endo conformation appears to be less dependent upon electron correlation as revealed by the MP2/6-31G*//HF/6-31G* values which also predict the same difference in energy. The opposite trend is noted for riboguanosine. At the HF/6-31G* and MP2/6-31G*//HF/6-31G* levels, the C3'-endo form of riboguanosine is shown to be about 0.65 and 1.41 kcal/mol more stable than the C2'endo form, respectively.

20

Table 1

Relative energies* of the C3'-endo and C2'-endo conformations of representative nucleosides.

| | HF/6-31G | MP2/6-31-G | CONTINUUM MODEL | AMBER |
|-------------|----------|------------|--------------------|-------|
| 25 dG | 0.60 | 0.56 | 0.88 | 0.65 |
| rG | -0.65 | -1.41 | -0.28 | -2.09 |
| 2'-O-MeG | -0.89 | -1.79 | -0.36 | -0.86 |
| 30 2'-S-MeG | 2.55 | 1.41 | 3.16 | 2.43 |

* energies are in kcal/mol relative to the C2'-endo conformation

Table 1 also includes the relative energies of 2'-O-methylguanosine and 2'-S-methylguanosine in C2'-endo and C3'-endo conformation. This data indicates the electronic nature of C2'-substitution has a significant impact on the relative stability of these conformations. Substitution of the 2'-O-methyl group increases the preference for the C3'-endo conformation (when compared to riboguanosine) by about 0.4 kcal/mol at both the HF/6-31G* and MP2/6-31G**/HF/6-31G* levels. In contrast, the 2'-S-methyl group reverses the trend. The C2'-endo conformation is favored by about 2.6 kcal/mol at the HF/6-31G* level, while the same difference is reduced to 1.41 kcal/mol at the MP2/6-31G**/HF/6-31G* level. For comparison, and also to evaluate the accuracy of the molecular mechanical force-field parameters used for the 2'-O-methyl and 2'-S-methyl substituted nucleosides, we have calculated the gas phase energies of the nucleosides. The results reported in Table 1 indicate that the calculated relative energies of these nucleosides compare qualitatively well with the *ab initio* calculations.

Additional calculations were also performed to gauge the effect of solvation on the relative stability of nucleoside conformations. The estimated solvation effect using HF/6-31G* geometries confirms that the relative energetic preference of the four nucleosides in the gas-phase is maintained in the aqueous phase as well (Table 1). Solvation effects were also examined using molecular dynamics simulations of the nucleosides in explicit water. From these trajectories, one can observe the predominance of C2'-endo conformation for deoxyriboguanosine and 2'-S-methylriboguanosine while riboguanosine and 2'-O-methylriboguanosine prefer the C3'-endo conformation. These results are in much accord with the available NMR results on 2'-S-methylribonucleosides. NMR studies of sugar puckering equilibrium using vicinal spin-coupling constants have indicated that the conformation of the sugar ring in 2'-S-methylpyrimidine nucleosides show an average of >75% S-character, whereas the corresponding purine analogs exhibit an average of >90% S-pucker [Fraser, A., Wheeler, P., Cook, P.D. and Sanghvi, Y.S., *J. Heterocycl. Chem.*, 1993, 30, 1277-1287]. It was observed that the 2'-S-methyl substitution in deoxynucleoside confers more conformational rigidity to the sugar conformation when compared with deoxyribonucleosides.

Structural features of DNA:RNA, OMe-DNA:RNA and SMe-DNA:RNA hybrids were also observed. The average RMS deviation of the DNA:RNA structure from the starting hybrid coordinates indicate the structure is stabilized over the length of the

simulation with an approximate average RMS deviation of 1.0 Å. This deviation is due, in part, to inherent differences in averaged structures (i.e. the starting conformation) and structures at thermal equilibrium. The changes in sugar pucker conformation for three of the central base pairs of this hybrid are in good agreement with the observations made in
5 previous NMR studies. The sugars in the RNA strand maintain very stable geometries in the C3'-endo conformation with ring pucker values near 0°. In contrast, the sugars of the DNA strand show significant variability.

The average RMS deviation of the OMe-DNA:RNA is approximately 1.2 Å from the starting A-form conformation; while the SMe-DNA:RNA shows a slightly higher
10 deviation (approximately 1.8 Å) from the starting hybrid conformation. The SMe-DNA strand also shows a greater variance in RMS deviation, suggesting the S-methyl group may induce some structural fluctuations. The sugar puckers of the RNA complements maintain C3'-endo puckering throughout the simulation. As expected from the nucleoside calculations, however, significant differences are noted in the puckering of the OMe-DNA
15 and SMe-DNA strands, with the former adopting C3'-endo, and the latter, C1'-exo/C2'-endo conformations.

An analysis of the helicoidal parameters for all three hybrid structures has also been performed to further characterize the duplex conformation. Three of the more important axis-basepair parameters that distinguish the different forms of the duplexes, X-
20 displacement, propeller twist, and inclination, are reported in Table 2. Usually, an X-displacement near zero represents a B-form duplex; while a negative displacement, which is a direct measure of deviation of the helix from the helical axis, makes the structure appear more A-like in conformation. In A-form duplexes, these values typically vary from -4Å to -5Å. In comparing these values for all three hybrids, the SMe_DNA:RNA hybrid
25 shows the most deviation from the A-form value, the OMe_DNA:RNA shows the least, and the DNA:RNA is intermediate. A similar trend is also evident when comparing the inclination and propeller twist values with ideal A-form parameters. These results are further supported by an analysis of the backbone and glycosidic torsion angles of the hybrid structures. Glycosidic angles (X) of A-form geometries, for example, are typically
30 near -159° while B form values are near -102°. These angles are found to be -162°, -133°, and -108° for the OMe-DNA, DNA, and SMe-DNA strands, respectively. All RNA complements adopt an X angle close to -160°. In addition, "crankshaft" transitions were

- also noted in the backbone torsions of the central UpU steps of the RNA strand in the SMe-DNA:RNA and DNA:RNA hybrids. Such transitions suggest some local conformational changes may occur to relieve a less favorable global conformation. Taken overall, the results indicate the amount of A-character decreases as OMe-
- 5 DNA:RNA>DNA:RNA>SMe-DNA:RNA, with the latter two adopting more intermediate conformations when compared to A- and B-form geometries.

Table 2

Average helical parameters derived from
the last 500 ps of simulation time.

10

(canonical A-and B-form values are given for comparison)

| Helicoidal Parameter | B-DNA (x-ray) | B-DNA (fibre) | A-DNA (fibre) | DNA:RNA | OMe_DNA: RNA | SMe_DNA: RNA |
|-------------------------|------------------|------------------|------------------|---------|-----------------|-----------------|
| X-disp | 1.2 | 0.0 | -5.3 | -4.5 | -5.4 | -3.5 |
| Inclination | -2.3 | 1.5 | 20.7 | 11.6 | 15.1 | 0.7 |
| Propeller | -16.4 | -13.3 | -7.5 | -12.7 | -15.8 | -10.3 |

- Stability of C2'-modified DNA:RNA hybrids was determined. Although the overall stability of the DNA:RNA hybrids depends on several factors including sequence-
- 15 dependencies and the purine content in the DNA or RNA strands DNA:RNA hybrids are usually less stable than RNA:RNA duplexes and, in some cases, even less stable than DNA:DNA duplexes. Available experimental data attributes the relatively lowered stability of DNA:RNA hybrids largely to its intermediate conformational nature between DNA:DNA (B-family) and RNA:RNA (A-family) duplexes. The overall thermodynamic
- 20 stability of nucleic acid duplexes may originate from several factors including the conformation of backbone, base-pairing and stacking interactions. While it is difficult to ascertain the individual thermodynamic contributions to the overall stabilization of the duplex, it is reasonable to argue that the major factors that promote increased stability of hybrid duplexes are better stacking interactions (electrostatic π - π interactions) and more
- 25 favorable groove dimensions for hydration. The C2'-S-methyl substitution has been shown to destabilize the hybrid duplex. The notable differences in the rise values among the three hybrids may offer some explanation. While the 2'-S-methyl group has a strong

influence on decreasing the base-stacking through high rise values (~ 3.2 Å), the 2'-O-methyl group makes the overall structure more compact with a rise value that is equal to that of A-form duplexes (~ 2.6 Å). Despite its overall A-like structural features, the SMe_DNA:RNA hybrid structure possesses an average rise value of 3.2 Å which is quite
 5 close to that of B-family duplexes. In fact, some local base-steps (CG steps) may be observed to have unusually high rise values (as high as 4.5 Å). Thus, the greater destabilization of 2'-S-methyl substituted DNA:RNA hybrids may be partly attributed to poor stacking interactions.

It has been postulated that RNase H binds to the minor groove of RNA:DNA
 10 hybrid complexes, requiring an intermediate minor groove width between ideal A- and B-form geometries to optimize interactions between the sugar phosphate backbone atoms and RNase H. A close inspection of the averaged structures for the hybrid duplexes using computer simulations reveals significant variation in the minor groove width dimensions as shown in Table 3. Whereas the O-methyl substitution leads to a slight expansion of the
 15 minor groove width when compared to the standard DNA:RNA complex, the S-methyl substitution leads to a general contraction (approximately 0.9 Å). These changes are most likely due to the preferred sugar puckering noted for the antisense strands which induce either A- or B-like single strand conformations. In addition to minor groove variations, the results also point to potential differences in the steric makeup of the minor groove.
 20 The O-methyl group points into the minor groove while the S-methyl is directed away towards the major groove. Essentially, the S-methyl group has flipped through the bases into the major groove as a consequence of C2'-endo puckering.

Table 3

25

| Minor groove widths averaged over the last 500 ps of simulation time | | | | | |
|---|---------|-----------------|-----------------|---------------------|---------------------|
| Phosphate Distance | DNA:RNA | OMe_DNA: RNA | SMe_DNA: RNA | DNA:RNA (B-form) | RNA:RNA (A-form) |
| P5-P20 | 15.27 | 16.82 | 13.73 | 14.19 | 17.32 |
| P6-P19 | 15.52 | 16.79 | 15.73 | 12.66 | 17.12 |
| P7-P18 | 15.19 | 16.40 | 14.08 | 11.10 | 16.60 |

| | | | | | |
|---------|-------|-------|-------|-------|-------|
| P8-P17 | 15.07 | 16.12 | 14.00 | 10.98 | 16.14 |
| P9-P16 | 15.29 | 16.25 | 14.98 | 11.65 | 16.93 |
| P10-P15 | 15.37 | 16.57 | 13.92 | 14.05 | 17.69 |

Chemistries Defined

Unless otherwise defined herein, alkyl means C₁-C₁₂, preferably C₁-C₈, and more preferably C₁-C₆, straight or (where possible) branched chain aliphatic hydrocarbyl.

- 5 Unless otherwise defined herein, heteroalkyl means C₁-C₁₂, preferably C₁-C₈, and more preferably C₁-C₆, straight or (where possible) branched chain aliphatic hydrocarbyl containing at least one, and preferably about 1 to about 3, hetero atoms in the chain, including the terminal portion of the chain. Preferred heteroatoms include N, O and S.

- 10 Unless otherwise defined herein, cycloalkyl means C₃-C₁₂, preferably C₃-C₈, and more preferably C₃-C₆, aliphatic hydrocarbyl ring.

Unless otherwise defined herein, alkenyl means C₂-C₁₂, preferably C₂-C₈, and more preferably C₂-C₆ alkenyl, which may be straight or (where possible) branched hydrocarbyl moiety, which contains at least one carbon-carbon double bond.

- 15 Unless otherwise defined herein, alkynyl means C₂-C₁₂, preferably C₂-C₈, and more preferably C₂-C₆ alkynyl, which may be straight or (where possible) branched hydrocarbyl moiety, which contains at least one carbon-carbon triple bond.

- 20 Unless otherwise defined herein, heterocycloalkyl means a ring moiety containing at least three ring members, at least one of which is carbon, and of which 1, 2 or three ring members are other than carbon. Preferably the number of carbon atoms varies from 1 to about 12, preferably 1 to about 6, and the total number of ring members varies from three to about 15, preferably from about 3 to about 8. Preferred ring heteroatoms are N, O and S. Preferred heterocycloalkyl groups include morpholino, thiomorpholino, piperidinyl, piperazinyl, homopiperidinyl, homopiperazinyl, homomorpholino, homothiomorpholino, pyrrolodinyl, tetrahydrooxazolyl, tetrahydroimidazolyl, tetrahydrothiazolyl, tetrahydroisoxazolyl, tetrahydropyrrazolyl, furanyl, pyranal, and tetrahydroisothiazolyl.

25 Unless otherwise defined herein, aryl means any hydrocarbon ring structure containing at least one aryl ring. Preferred aryl rings have about 6 to about 20 ring carbons. Especially preferred aryl rings include phenyl, naphthyl, anthracenyl, and phenanthrenyl.

Unless otherwise defined herein, hetaryl means a ring moiety containing at least one fully unsaturated ring, the ring consisting of carbon and non-carbon atoms. Preferably the ring system contains about 1 to about 4 rings. Preferably the number of carbon atoms varies from 1 to about 12, preferably 1 to about 6, and the total number of ring members
5 varies from three to about 15, preferably from about 3 to about 8. Preferred ring heteroatoms are N, O and S. Preferred hetaryl moieties include pyrazolyl, thiophenyl, pyridyl, imidazolyl, tetrazolyl, pyridyl, pyrimidinyl, purinyl, quinazolinyl, quinoxalinyl, benzimidazolyl, benzothiophenyl, etc.

Unless otherwise defined herein, where a moiety is defined as a compound moiety,
10 such as hetarylalkyl (hetaryl and alkyl), aralkyl (aryl and alkyl), etc., each of the sub-moieties is as defined herein.

Unless otherwise defined herein, an electron withdrawing group is a group, such as the cyano or isocyanato group that draws electronic charge away from the carbon to which it is attached. Other electron withdrawing groups of note include those whose
15 electronegativities exceed that of carbon, for example halogen, nitro, or phenyl substituted in the ortho- or para-position with one or more cyano, isothiocyanato, nitro or halo groups.

Unless otherwise defined herein, the terms halogen and halo have their ordinary meanings. Preferred halo (halogen) substituents are Cl, Br, and I.

The aforementioned optional substituents are, unless otherwise herein defined,
20 suitable substituents depending upon desired properties. Included are halogens (Cl, Br, I), alkyl, alkenyl, and alkynyl moieties, NO₂, NH₃ (substituted and unsubstituted), acid moieties (e.g. -CO₂H, -OSO₃H₂, etc.), heterocycloalkyl moieties, hetaryl moieties, aryl moieties, etc.

In all the preceding formulae, the squiggle (~) indicates a bond to an oxygen or
25 sulfur of the 5'-phosphate. Phosphate protecting groups include those described in US Patents No. US 5,760,209, US 5,614,621, US 6,051,699, US 6,020,475, US 6,326,478, US 6,169,177, US 6,121,437, US 6,465,628 each of which is expressly incorporated herein by reference in its entirety.

30 **Oligomer Synthesis**

Oligomerization of modified and unmodified nucleosides is performed according to literature procedures for DNA (Protocols for Oligonucleotides and Analogs, Ed.

Agrawal (1993), Humana Press) and/or RNA (Scaringe, Methods (2001), 23, 206-217. Gait et al., Applications of Chemically synthesized RNA in RNA:Protein Interactions, Ed. Smith (1998), 1-36. Gallo et al., Tetrahedron (2001), 57, 5707-5713) synthesis as appropriate. In addition specific protocols for the synthesis of oligomeric compounds of
5 the invention are illustrated in the examples below.

The oligomeric compounds used in accordance with this invention may be conveniently and routinely made through the well-known technique of solid phase synthesis. Equipment for such synthesis is sold by several vendors including, for example, Applied Biosystems (Foster City, CA). Any other means for such synthesis known in the
10 art may additionally or alternatively be employed. It is well known to use similar techniques to prepare oligonucleotides such as the phosphorothioates and alkylated derivatives.

The present invention is also useful for the preparation of oligomeric compounds incorporating at least one 2'-O-protected nucleoside. After incorporation and appropriate
15 deprotection the 2'-O-protected nucleoside will be converted to a ribonucleoside at the position of incorporation. The number and position of the 2-ribonucleoside units in the final oligomeric compound can vary from one at any site or the strategy can be used to prepare up to a full 2'-OH modified oligomeric compound. All 2'-O-protecting groups amenable to the synthesis of oligomeric compounds are included in the present invention.
20 In general a protected nucleoside is attached to a solid support by for example a succinate linker. Then the oligonucleotide is elongated by repeated cycles of deprotecting the 5'-terminal hydroxyl group, coupling of a further nucleoside unit, capping and oxidation (alternatively sulfurization). In a more frequently used method of synthesis the completed oligonucleotide is cleaved from the solid support with the removal of phosphate protecting
25 groups and exocyclic amino protecting groups by treatment with an ammonia solution. Then a further deprotection step is normally required for removal of the more specialized protecting groups used for the protection of 2'-hydroxyl groups thereby affording the fully deprotected oligonucleotide.

A large number of 2'-O-protecting groups have been used for the synthesis of
30 oligoribonucleotides but over the years more effective groups have been discovered. The key to an effective 2'-O-protecting group is that it is capable of selectively being introduced at the 2'-O-position and that it can be removed easily after synthesis without

the formation of unwanted side products. The protecting group also needs to be inert to the normal deprotecting, coupling, and capping steps required for oligoribonucleotide synthesis. Some of the protecting groups used initially for oligoribonucleotide synthesis included tetrahydropyran-1-yl and 4-methoxytetrahydropyran-4-yl. These two groups are not compatible with all 5'-O-protecting groups so modified versions were used with 5'-DMT groups such as 1-(2-fluorophenyl)-4-methoxypiperidin-4-yl (Fpmp). Reese has identified a number of piperidine derivatives (like Fpmp) that are useful in the synthesis of oligoribonucleotides including 1-[(chloro-4-methyl)phenyl]-4'-methoxypiperidin-4-yl (Reese et al., *Tetrahedron Lett.*, 1986, (27), 2291). Another approach was to replace the standard 5'-DMT (dimethoxytrityl) group with protecting groups that were removed under non-acidic conditions such as levulinyl and 9-fluorenylmethoxycarbonyl. Such groups enable the use of acid labile 2'-protecting groups for oligoribonucleotide synthesis. Another more widely used protecting group initially used for the synthesis of oligoribonucleotides was the t-butyldimethylsilyl group (Ogilvie et al., *Tetrahedron Lett.*, 1974, 2861; Hakimelahi et al., *Tetrahedron Lett.*, 1981, (22), 2543; and Jones et al., *J. Chem. Soc. Perkin I.*, 2762). The 2'-O-protecting groups can require special reagents for their removal such as for example the t-butyldimethylsilyl group is normally removed after all other cleaving/deprotecting steps by treatment of the oligomeric compound with tetrabutylammonium fluoride (TBAF).

One group of researchers examined a number of 2'-O-protecting groups (Pitsch, S., *Chimia*, 2001, (55), 320-324.) The group examined fluoride labile and photolabile protecting groups that are removed using moderate conditions. One photolabile group that was examined was the [2-(nitrobenzyl)oxy]methyl (nbm) protecting group (Schwartz et al., *Bioorg. Med. Chem. Lett.*, 1992, (2), 1019.) Other groups examined included a number structurally related formaldehyde acetal-derived, 2'-O-protecting groups. Also prepared were a number of related protecting groups for preparing 2'-O-alkylated nucleoside phosphoramidites including 2'-O-[(triisopropylsilyl)oxy]methyl (2'-O-CH₂-O-Si(iPr)₃, TOM). One 2'-O-protecting group that was prepared to be used orthogonally to the TOM group was 2'-O-[(R)-1-(2-nitrophenyl)ethyloxy]methyl ((R)-mnbm).

Another strategy using a fluoride labile 5'-O-protecting group (non-acid labile) and an acid labile 2'-O-protecting group has been reported (Scaringe, Stephen A., *Methods*, 2001, (23) 206-217). A number of possible silyl ethers were examined for 5'-O-protection

and a number of acetals and orthoesters were examined for 2'-O-protection. The protection scheme that gave the best results was 5'-O-silyl ether-2'-ACE (5'-O-bis(trimethylsiloxy)cyclododecyloxysilyl ether (DOD)-2'-O-bis(2-acetoxyethoxy)methyl (ACE). This approach uses a modified phosphoramidite synthesis approach in that some
5 different reagents are required that are not routinely used for RNA/DNA synthesis.

Although a lot of research has focused on the synthesis of oligoribonucleotides the main RNA synthesis strategies that are presently being used commercially include 5'-O-DMT-2'-O-t-butyl dimethylsilyl (TBDMS), 5'-O-DMT-2'-O-[1(2-fluorophenyl)-4-methoxypiperidin-4-yl] (FPMP), 2'-O-[(triisopropylsilyl)oxy]methyl (2'-O-CH₂-O-Si(iPr)₃) (TOM), and the 5'-O-silyl ether-2'-ACE (5'-O-bis(trimethylsiloxy)cyclododecyloxysilyl ether (DOD)-2'-O-bis(2-acetoxyethoxy)methyl (ACE). A current list of some of the major companies currently offering RNA products include Pierce Nucleic Acid Technologies, Dharmacon Research Inc., Ameri Biotechnologies Inc., and Integrated DNA Technologies, Inc. One company, Princeton Separations, is marketing an RNA synthesis
10 activator advertised to reduce coupling times especially with TOM and TBDMS chemistries. Such an activator would also be amenable to the present invention.

The primary groups being used for commercial RNA synthesis are:

TBDMS = 5'-O-DMT-2'-O-t-butyl dimethylsilyl;
TOM = 2'-O-[(triisopropylsilyl)oxy]methyl;
20 DOD/ACE = (5'-O-bis(trimethylsiloxy)cyclododecyloxysilyl ether-2'-O-bis(2-acetoxyethoxy)methyl
FPMP = 5'-O-DMT-2'-O-[1(2-fluorophenyl)-4-methoxypiperidin-4-yl] .

All of the aforementioned RNA synthesis strategies are amenable to the present invention. Strategies that would be a hybrid of the above e.g. using a 5'-protecting group
25 from one strategy with a 2'-O-protecting from another strategy is also amenable to the present invention.

The preparation of ribonucleotides and oligomeric compounds having at least one ribonucleoside incorporated and all the possible configurations falling in between these two extremes are encompassed by the present invention. The corresponding oligomeric
30 compounds can be hybridized to further oligomeric compounds including oligoribonucleotides having regions of complementarity to form double-stranded (duplexed) oligomeric compounds. Such double stranded oligonucleotide moieties have

been shown in the art to modulate target expression and regulate translation as well as RNA processing via an antisense mechanism. Moreover, the double-stranded moieties may be subject to chemical modifications (Fire et al., *Nature*, 1998, 391, 806-811; Timmons and Fire, *Nature* 1998, 395, 854; Timmons et al., *Gene*, 2001, 263, 103-112; 5 Tabara et al., *Science*, 1998, 282, 430-431; Montgomery et al., *Proc. Natl. Acad. Sci. USA*, 1998, 95, 15502-15507; Tuschl et al., *Genes Dev.*, 1999, 13, 3191-3197; Elbashir et al., *Nature*, 2001, 411, 494-498; Elbashir et al., *Genes Dev.* 2001, 15, 188-200). For example, such double-stranded moieties have been shown to inhibit the target by the classical hybridization of antisense strand of the duplex to the target, thereby triggering 10 enzymatic degradation of the target (Tijsterman et al., *Science*, 2002, 295, 694-697).

The methods of preparing oligomeric compounds of the present invention can also be applied in the areas of drug discovery and target validation. The present invention comprehends the use of the oligomeric compounds and preferred targets identified herein in drug discovery efforts to elucidate relationships that exist between proteins and a 15 disease state, phenotype, or condition. These methods include detecting or modulating a target peptide comprising contacting a sample, tissue, cell, or organism with the oligomeric compounds of the present invention, measuring the nucleic acid or protein level of the target and/or a related phenotypic or chemical endpoint at some time after treatment, and optionally comparing the measured value to a non-treated sample or sample 20 treated with a further oligomeric compound of the invention. These methods can also be performed in parallel or in combination with other experiments to determine the function of unknown genes for the process of target validation or to determine the validity of a particular gene product as a target for treatment or prevention of a particular disease, condition, or phenotype.

25 Effect of nucleoside modifications on RNAi activity is evaluated according to existing literature (Elbashir et al., *Nature* (2001), 411, 494-498; Nishikura et al., *Cell* (2001), 107, 415-416; and Bass et al., *Cell* (2000), 101, 235-238.)

Targets of the invention

30 "Targeting" an antisense oligomeric compound to a particular nucleic acid molecule, in the context of this invention, can be a multistep process. The process usually begins with the identification of a target nucleic acid whose function is to be modulated.

This target nucleic acid may be, for example, a cellular gene (or mRNA transcribed from the gene) whose expression is associated with a particular disorder or disease state, or a nucleic acid molecule from an infectious agent.

The targeting process usually also includes determination of at least one target
5 region, segment, or site within the target nucleic acid for the antisense interaction to occur such that the desired effect, e.g., modulation of expression, will result. Within the context of the present invention, the term "region" is defined as a portion of the target nucleic acid having at least one identifiable structure, function, or characteristic. Within regions of target nucleic acids are segments. "Segments" are defined as smaller or sub-portions of
10 regions within a target nucleic acid. "Sites," as used in the present invention, are defined as positions within a target nucleic acid. The terms region, segment, and site can also be used to describe an oligomeric compound of the invention such as for example a gapped oligomeric compound having 3 separate segments.

Since, as is known in the art, the translation initiation codon is typically 5'-AUG
15 (in transcribed mRNA molecules; 5'-ATG in the corresponding DNA molecule), the translation initiation codon is also referred to as the "AUG codon," the "start codon" or the "AUG start codon". A minority of genes have a translation initiation codon having the RNA sequence 5'-GUG, 5'-UUG or 5'-CUG, and 5'-AUA, 5'-ACG and 5'-CUG have been shown to function *in vivo*. Thus, the terms "translation initiation codon" and "start codon"
20 can encompass many codon sequences, even though the initiator amino acid in each instance is typically methionine (in eukaryotes) or formylmethionine (in prokaryotes). It is also known in the art that eukaryotic and prokaryotic genes may have two or more alternative start codons, any one of which may be preferentially utilized for translation initiation in a particular cell type or tissue, or under a particular set of conditions. In the
25 context of the invention, "start codon" and "translation initiation codon" refer to the codon or codons that are used *in vivo* to initiate translation of an mRNA transcribed from a gene encoding a nucleic acid target, regardless of the sequence(s) of such codons. It is also known in the art that a translation termination codon (or "stop codon") of a gene may have one of three sequences, i.e., 5'-UAA, 5'-UAG and 5'-UGA (the corresponding DNA
30 sequences are 5'-TAA, 5'-TAG and 5'-TGA, respectively).

The terms "start codon region" and "translation initiation codon region" refer to a portion of such an mRNA or gene that encompasses from about 25 to about 50 contiguous

nucleotides in either direction (i.e., 5' or 3') from a translation initiation codon. Similarly, the terms "stop codon region" and "translation termination codon region" refer to a portion of such an mRNA or gene that encompasses from about 25 to about 50 contiguous nucleotides in either direction (i.e., 5' or 3') from a translation termination codon.

- 5 Consequently, the "start codon region" (or "translation initiation codon region") and the "stop codon region" (or "translation termination codon region") are all regions which may be targeted effectively with the antisense oligomeric compounds of the present invention.

The open reading frame (ORF) or "coding region," which is known in the art to refer to the region between the translation initiation codon and the translation termination
10 codon, is also a region which may be targeted effectively. Within the context of the present invention, a preferred region is the intragenic region encompassing the translation initiation or termination codon of the open reading frame (ORF) of a gene.

Other target regions include the 5' untranslated region (5'UTR), known in the art to refer to the portion of an mRNA in the 5' direction from the translation initiation codon,
15 and thus including nucleotides between the 5' cap site and the translation initiation codon of an mRNA (or corresponding nucleotides on the gene), and the 3' untranslated region (3'UTR), known in the art to refer to the portion of an mRNA in the 3' direction from the translation termination codon, and thus including nucleotides between the translation termination codon and 3' end of an mRNA (or corresponding nucleotides on the gene).

- 20 The 5' cap site of an mRNA comprises an N7-methylated guanosine residue joined to the 5'-most residue of the mRNA via a 5'-5' triphosphate linkage. The 5' cap region of an mRNA is considered to include the 5' cap structure itself as well as the first 50 nucleotides adjacent to the cap site. It is also preferred to target the 5' cap region.

Although some eukaryotic mRNA transcripts are directly translated, many contain
25 one or more regions, known as "introns," which are excised from a transcript before it is translated. The remaining (and therefore translated) regions are known as "exons" and are spliced together to form a continuous mRNA sequence. Targeting splice sites, i.e., intron-exon junctions or exon-intron junctions, may also be particularly useful in situations where aberrant splicing is implicated in disease, or where an overproduction of a particular splice
30 product is implicated in disease. Aberrant fusion junctions due to rearrangements or deletions are also preferred target sites. mRNA transcripts produced via the process of splicing of two (or more) mRNAs from different gene sources are known as "fusion

transcripts". It is also known that introns can be effectively targeted using antisense oligomeric compounds targeted to, for example, DNA or pre-mRNA.

It is also known in the art that alternative RNA transcripts can be produced from the same genomic region of DNA. These alternative transcripts are generally known as "variants". More specifically, "pre-mRNA variants" are transcripts produced from the same genomic DNA that differ from other transcripts produced from the same genomic DNA in either their start or stop position and contain both intronic and exonic sequences.

Upon excision of one or more exon or intron regions, or portions thereof during splicing, pre-mRNA variants produce smaller "mRNA variants". Consequently, mRNA variants are processed pre-mRNA variants and each unique pre-mRNA variant must always produce a unique mRNA variant as a result of splicing. These mRNA variants are also known as "alternative splice variants". If no splicing of the pre-mRNA variant occurs then the pre-mRNA variant is identical to the mRNA variant.

It is also known in the art that variants can be produced through the use of alternative signals to start or stop transcription and that pre-mRNAs and mRNAs can possess more than one start codon or stop codon. Variants that originate from a pre-mRNA or mRNA that use alternative start codons are known as "alternative start variants" of that pre-mRNA or mRNA. Those transcripts that use an alternative stop codon are known as "alternative stop variants" of that pre-mRNA or mRNA. One specific type of alternative stop variant is the "polyA variant" in which the multiple transcripts produced result from the alternative selection of one of the "polyA stop signals" by the transcription machinery, thereby producing transcripts that terminate at unique polyA sites. Within the context of the invention, the types of variants described herein are also preferred target nucleic acids.

The locations on the target nucleic acid to which the preferred antisense oligomeric compounds hybridize are hereinbelow referred to as "preferred target segments." As used herein the term "preferred target segment" is defined as at least an 8-nucleobase portion of a target region to which an active antisense oligomeric compound is targeted. While not wishing to be bound by theory, it is presently believed that these target segments represent accessible portions of the target nucleic acid for hybridization.

Exemplary preferred antisense oligomeric compounds include oligomeric compounds that comprise at least the 8 consecutive nucleobases from the 5'-terminus of a

targeted nucleic acid e.g. a cellular gene or mRNA transcribed from the gene (the remaining nucleobases being a consecutive stretch of the same oligonucleotide beginning immediately upstream of the 5'-terminus of the antisense compound which is specifically hybridizable to the target nucleic acid and continuing until the oligonucleotide contains

5 from about 8 to about 80 nucleobases). Similarly preferred antisense oligomeric compounds are represented by oligonucleotide sequences that comprise at least the 8 consecutive nucleobases from the 3'-terminus of one of the illustrative preferred antisense compounds (the remaining nucleobases being a consecutive stretch of the same oligonucleotide beginning immediately downstream of the 3'-terminus of the antisense

10 compound which is specifically hybridizable to the target nucleic acid and continuing until the oligonucleotide contains from about 8 to about 80 nucleobases). One having skill in the art armed with the preferred antisense compounds illustrated herein will be able, without undue experimentation, to identify further preferred antisense compounds.

Once one or more target regions, segments or sites have been identified, antisense

15 oligomeric compounds are chosen which are sufficiently complementary to the target, i.e., hybridize sufficiently well and with sufficient specificity, to give the desired effect.

In accordance with one embodiment of the present invention, a series of preferred compositions of nucleic acid duplexes comprising the antisense oligomeric compounds of the present invention and their complements can be designed for a specific target or

20 targets. The ends of the strands may be modified by the addition of one or more natural or modified nucleobases to form an overhang. The sense strand of the duplex is then designed and synthesized as the complement of the antisense strand and may also contain modifications or additions to either terminus. For example, in one embodiment, both strands of the duplex would be complementary over the central nucleobases, each having

25 overhangs at one or both termini.

For example, a duplex comprising an antisense oligomeric compound having the sequence CGAGAGGCGGACGGGACCG and having a two-nucleobase overhang of deoxythymidine(dT) would have the following structure:

30

| | | |
|--|--------------------------------|--------------------------|
| | cgagaggcggacgggaccgdTdT | Antisense Strand |
| | | |
| | dTdTgctctccgcctgccctggc | Complement Strand |

RNA strands of the duplex can be synthesized by methods disclosed herein or purchased from various RNA synthesis companies such as for example Dharmacon

Research Inc., (Lafayette, CO). Once synthesized, the complementary strands are annealed. The single strands are aliquoted and diluted to a concentration of 50 uM. Once diluted, 30 uL of each strand is combined with 15uL of a 5X solution of annealing buffer. The final concentration of the buffer is 100 mM potassium acetate, 30 mM HEPES-KOH
5 pH 7.4, and 2mM magnesium acetate. The final volume is 75 uL. This solution is incubated for 1 minute at 90°C and then centrifuged for 15 seconds. The tube is allowed to sit for 1 hour at 37°C at which time the dsRNA duplexes are used in experimentation. The final concentration of the dsRNA compound is 20 uM. This solution can be stored frozen (-20°C) and freeze-thawed up to 5 times.

10 Once prepared, the desired synthetic duplexs are evaluated for their ability to modulate target expression. When cells reach 80% confluency, they are treated with synthetic duplexs comprising at least one oligomeric compound of the invention. For cells grown in 96-well plates, wells are washed once with 200 µL OPTI-MEM-1 reduced-serum medium (Gibco BRL) and then treated with 130 µL of OPTI-MEM-1 containing 12
15 µg/mL LIPOFECTIN (Gibco BRL) and the desired dsRNA compound at a final concentration of 200 nM. After 5 hours of treatment, the medium is replaced with fresh medium. Cells are harvested 16 hours after treatment, at which time RNA is isolated and target reduction measured by RT-PCR.

In a further embodiment, the "preferred target segments" identified herein may be
20 employed in a screen for additional oligomeric compounds that modulate the expression of a target. "Modulators" are those oligomeric compounds that decrease or increase the expression of a nucleic acid molecule encoding a target and which comprise at least an 8-nucleobase portion which is complementary to a preferred target segment. The screening method comprises the steps of contacting a preferred target segment of a nucleic acid
25 molecule encoding a target with one or more candidate modulators, and selecting for one or more candidate modulators which decrease or increase the expression of a nucleic acid molecule encoding a target. Once it is shown that the candidate modulator or modulators are capable of modulating (e.g. either decreasing or increasing) the expression of a nucleic acid molecule encoding a target, the modulator may then be employed in further
30 investigative studies of the function of a target, or for use as a research, diagnostic, or therapeutic agent in accordance with the present invention.

The preferred target segments of the present invention may also be combined with their respective complementary antisense oligomeric compounds of the present invention to form stabilized double-stranded (duplexed) oligonucleotides.

5 **Hybridization**

In the context of this invention, "hybridization" occurs when two sequences come together with enough base complementarity to form a double stranded region. The source of the two sequences can be synthetic or native and can occur in a single strand when the strand has regions of self complementarity. In the present invention, the preferred
10 mechanism of pairing involves hydrogen bonding, which may be Watson-Crick, Hoogsteen or reversed Hoogsteen hydrogen bonding, between complementary nucleoside or nucleotide bases (nucleobases) of the strands of oligomeric compounds or between an oligomeric compound and a target nucleic acid. For example, adenine and thymine are complementary nucleobases which pair through the formation of hydrogen bonds.

15 Hybridization can occur under varying circumstances.

An antisense oligomeric compound is specifically hybridizable when binding of the compound to the target nucleic acid interferes with the normal function of the target nucleic acid to cause a loss of activity, and there is a sufficient degree of complementarity to avoid non-specific binding of the antisense oligomeric compound to non-target nucleic
20 acid sequences under conditions in which specific binding is desired, i.e., under physiological conditions in the case of *in vivo* assays or therapeutic treatment, and under conditions in which assays are performed in the case of *in vitro* assays.

In the present invention the phrase "stringent hybridization conditions" or "stringent conditions" refers to conditions under which an oligomeric compound of the
25 invention will hybridize to its target sequence, but to a minimal number of other sequences. Stringent conditions are sequence-dependent and will vary with different circumstances and in the context of this invention, "stringent conditions" under which oligomeric compounds hybridize to a target sequence are determined by the nature and composition of the oligomeric compounds and the assays in which they are being
30 investigated.

"Complementary," as used herein, refers to the capacity for precise pairing of two nucleobases regardless of where the two are located. For example, if a nucleobase at a

certain position of an oligomeric compound is capable of hydrogen bonding with a nucleobase at a certain position of a target nucleic acid, the target nucleic acid being a DNA, RNA, or oligonucleotide molecule, then the position of hydrogen bonding between the oligonucleotide and the target nucleic acid is considered to be a complementary position. The oligomeric compound and the further DNA, RNA, or oligonucleotide molecule are complementary to each other when a sufficient number of complementary positions in each molecule are occupied by nucleobases which can hydrogen bond with each other. Thus, "specifically hybridizable" and "complementary" are terms which are used to indicate a sufficient degree of precise pairing or complementarity over a sufficient number of nucleobases such that stable and specific binding occurs between the oligonucleotide and a target nucleic acid.

It is understood in the art that the sequence of an antisense oligomeric compound need not be 100% complementary to that of its target nucleic acid to be specifically hybridizable. Moreover, an oligonucleotide may hybridize over one or more segments such that intervening or adjacent segments are not involved in the hybridization event (e.g., a loop structure or hairpin structure). It is preferred that the antisense oligomeric compounds of the present invention comprise at least 70% sequence complementarity to a target region within the target nucleic acid, more preferably that they comprise 90% sequence complementarity and even more preferably comprise 95% sequence complementarity to the target region within the target nucleic acid sequence to which they are targeted. For example, an antisense oligomeric compound in which 18 of 20 nucleobases of the antisense oligomeric compound are complementary to a target region, and would therefore specifically hybridize, would represent 90 percent complementarity. In this example, the remaining noncomplementary nucleobases may be clustered or interspersed with complementary nucleobases and need not be contiguous to each other or to complementary nucleobases. As such, an antisense oligomeric compound which is 18 nucleobases in length having 4 (four) noncomplementary nucleobases which are flanked by two regions of complete complementarity with the target nucleic acid would have 77.8% overall complementarity with the target nucleic acid and would thus fall within the scope of the present invention. Percent complementarity of an antisense oligomeric compound with a region of a target nucleic acid can be determined routinely using BLAST programs (basic local alignment search tools) and PowerBLAST programs known in the

art (Altschul et al., J. Mol. Biol., 1990, 215, 403-410; Zhang and Madden, Genome Res., 1997, 7, 649-656).

Screening and Target Validation

5 In a further embodiment, "preferred target segments" may be employed in a screen for additional oligomeric compounds that modulate the expression of a selected protein. "Modulators" are those oligomeric compounds that decrease or increase the expression of a nucleic acid molecule encoding a protein and which comprise at least an 8-nucleobase portion which is complementary to a preferred target segment. The screening method
10 comprises the steps of contacting a preferred target segment of a nucleic acid molecule encoding a protein with one or more candidate modulators, and selecting for one or more candidate modulators which decrease or increase the expression of a nucleic acid molecule encoding a protein. Once it is shown that the candidate modulator or modulators are capable of modulating (e.g. either decreasing or increasing) the expression of a nucleic
15 acid molecule encoding a peptide, the modulator may then be employed in further investigative studies of the function of the peptide, or for use as a research, diagnostic, or therapeutic agent in accordance with the present invention.

 The preferred target segments of the present invention may also be combined with their respective complementary antisense oligomeric compounds of the present
20 invention to form stabilized double-stranded (duplexed) oligonucleotides. Such double stranded oligonucleotide moieties have been shown in the art to modulate target expression and regulate translation as well as RNA processing via an antisense mechanism. Moreover, the double-stranded moieties may be subject to chemical modifications (Fire et al., *Nature*, **1998**, *391*, 806-811; Timmons and Fire, *Nature* **1998**,
25 395, 854; Timmons et al., *Gene*, **2001**, *263*, 103-112; Tabara et al., *Science*, **1998**, 282, 430-431; Montgomery et al., *Proc. Natl. Acad. Sci. USA*, **1998**, *95*, 15502-15507; Tuschl et al., *Genes Dev.*, **1999**, *13*, 3191-3197; Elbashir et al., *Nature*, **2001**, *411*, 494-498; Elbashir et al., *Genes Dev.* **2001**, *15*, 188-200). For example, such double-stranded moieties have been shown to inhibit the target by the classical hybridization of antisense
30 strand of the duplex to the target, thereby triggering enzymatic degradation of the target (Tijsterman et al., *Science*, **2002**, *295*, 694-697).

The compositions comprising oligomeric compounds of the present invention can also be applied in the areas of drug discovery and target validation. The present invention comprehends the use of the oligomeric compounds and preferred targets identified herein in drug discovery efforts to elucidate relationships that exist between proteins and a
5 disease state, phenotype, or condition. These methods include detecting or modulating a target peptide comprising contacting a sample, tissue, cell, or organism with the oligomeric compounds of the present invention, measuring the nucleic acid or protein level of the target and/or a related phenotypic or chemical endpoint at some time after treatment, and optionally comparing the measured value to a non-treated sample or sample
10 treated with a further oligomeric compound of the invention. These methods can also be performed in parallel or in combination with other experiments to determine the function of unknown genes for the process of target validation or to determine the validity of a particular gene product as a target for treatment or prevention of a particular disease, condition, or phenotype.

15 Effect of nucleoside modifications on RNAi activity is evaluated according to existing literature (Elbashir et al., Nature (2001), 411, 494-498; Nishikura et al., Cell (2001), 107, 415-416; and Bass et al., Cell (2000), 101, 235-238.)

Kits, Research Reagents, Diagnostics, and Therapeutics

20 The compositions of oligomeric compounds of the present invention can be utilized for diagnostics, therapeutics, prophylaxis and as research reagents and kits. Furthermore, antisense oligonucleotides, which are able to inhibit gene expression with exquisite specificity, are often used by those of ordinary skill to elucidate the function of particular genes or to distinguish between functions of various members of a biological
25 pathway.

For use in kits and diagnostics, the compositions of the present invention, either alone or in combination with other oligomeric compounds or therapeutics, can be used as tools in differential and/or combinatorial analyses to elucidate expression patterns of a portion or the entire complement of genes expressed within cells and tissues.

30 As one nonlimiting example, expression patterns within cells or tissues treated with one or more antisense oligomeric compounds are compared to control cells or tissues not treated with antisense oligomeric compounds and the patterns produced are analyzed

for differential levels of gene expression as they pertain, for example, to disease association, signaling pathway, cellular localization, expression level, size, structure or function of the genes examined. These analyses can be performed on stimulated or unstimulated cells and in the presence or absence of other compounds and or oligomeric
5 compounds that affect expression patterns.

Examples of methods of gene expression analysis known in the art include DNA arrays or microarrays (Brazma and Vilo, *FEBS Lett.*, **2000**, 480, 17-24; Celis, *et al.*, *FEBS Lett.*, **2000**, 480, 2-16), SAGE (serial analysis of gene expression)(Madden, *et al.*, *Drug Discov. Today*, **2000**, 5, 415-425), READS (restriction enzyme amplification of digested
10 cDNAs) (Prashar and Weissman, *Methods Enzymol.*, **1999**, 303, 258-72), TOGA (total gene expression analysis) (Sutcliffe, *et al.*, *Proc. Natl. Acad. Sci. U. S. A.*, **2000**, 97, 1976-81), protein arrays and proteomics (Celis, *et al.*, *FEBS Lett.*, **2000**, 480, 2-16; Jungblut, *et al.*, *Electrophoresis*, **1999**, 20, 2100-10), expressed sequence tag (EST) sequencing (Celis, *et al.*, *FEBS Lett.*, **2000**, 480, 2-16; Larsson, *et al.*, *J. Biotechnol.*, **2000**, 80, 143-57),
15 subtractive RNA fingerprinting (SuRF) (Fuchs, *et al.*, *Anal. Biochem.*, **2000**, 286, 91-98; Larson, *et al.*, *Cytometry*, **2000**, 41, 203-208), subtractive cloning, differential display (DD) (Jurecic and Belmont, *Curr. Opin. Microbiol.*, **2000**, 3, 316-21), comparative genomic hybridization (Carulli, *et al.*, *J. Cell Biochem. Suppl.*, **1998**, 31, 286-96), FISH (fluorescent *in situ* hybridization) techniques (Going and Gusterson, *Eur. J. Cancer*, **1999**,
20 35, 1895-904) and mass spectrometry methods (To, *Comb. Chem. High Throughput Screen*, **2000**, 3, 235-41).

The compositions of the invention are useful for research and diagnostics in one sense because the oligomeric compounds of the compositions hybridize to nucleic acids encoding proteins. For example, oligonucleotides that are shown to hybridize with such
25 efficiency and under such conditions as disclosed herein as to be effective protein inhibitors will also be effective primers or probes under conditions favoring gene amplification or detection, respectively. These primers and probes are useful in methods requiring the specific detection of nucleic acid molecules encoding proteins and in the amplification of the nucleic acid molecules for detection or for use in further studies.
30 Hybridization of the antisense oligonucleotides, particularly the primers and probes, of the invention with a nucleic acid can be detected by means known in the art. Such means may include conjugation of an enzyme to the oligonucleotide, radiolabelling of the

oligonucleotide or any other suitable detection means. Kits using such detection means for detecting the level of selected proteins in a sample may also be prepared.

The specificity and sensitivity of antisense methodologies is also harnessed by those of skill in the art for therapeutic uses. Antisense oligomeric compounds have been employed as therapeutic moieties in the treatment of disease states in animals, including humans. Antisense oligonucleotide drugs, including ribozymes, have been safely and effectively administered to humans and numerous clinical trials are presently underway. It is thus established that antisense oligomeric compounds can be useful therapeutic modalities that can be configured to be useful in treatment regimes for the treatment of cells, tissues and animals, especially humans.

For therapeutics, an animal, preferably a human, suspected of having a disease or disorder which can be treated by modulating the expression of a selected protein is treated by administering compositions of the invention in accordance with this invention. For example, in one non-limiting embodiment, the methods comprise the step of administering to the animal in need of treatment, a therapeutically effective amount of a protein inhibitor. The protein inhibitors of the present invention effectively inhibit the activity of the protein or inhibit the expression of the protein. In one embodiment, the activity or expression of a protein in an animal is inhibited by about 10%. Preferably, the activity or expression of a protein in an animal is inhibited by about 30%. More preferably, the activity or expression of a protein in an animal is inhibited by 50% or more.

For example, the reduction of the expression of a protein may be measured in serum, adipose tissue, liver or any other body fluid, tissue or organ of the animal. Preferably, the cells contained within the fluids, tissues or organs being analyzed contain a nucleic acid molecule encoding a protein and/or the protein itself.

The compositions of the invention can be utilized in pharmaceutical compositions by adding an effective amount to a suitable pharmaceutically acceptable diluent or carrier. Use of the compositions and methods of the invention may also be useful prophylactically.

Formulations

The compositions of the invention may also be admixed, encapsulated, conjugated or otherwise associated with other molecules, molecule structures or mixtures of compounds, as for example, liposomes, receptor-targeted molecules, oral, rectal, topical or

other formulations, for assisting in uptake, distribution and/or absorption. Representative United States patents that teach the preparation of such uptake, distribution and/or absorption-assisting formulations include, but are not limited to, U.S.: 5,108,921; 5,354,844; 5,416,016; 5,459,127; 5,521,291; 5,543,158; 5,547,932; 5,583,020; 5,591,721; 5 4,426,330; 4,534,899; 5,013,556; 5,108,921; 5,213,804; 5,227,170; 5,264,221; 5,356,633; 5,395,619; 5,416,016; 5,417,978; 5,462,854; 5,469,854; 5,512,295; 5,527,528; 5,534,259; 5,543,152; 5,556,948; 5,580,575; and 5,595,756, each of which is herein incorporated by reference.

The compositions of the invention encompass any pharmaceutically acceptable salts, esters, or salts of such esters, or any other compound which, upon administration to 10 an animal, including a human, is capable of providing (directly or indirectly) the biologically active metabolite or residue thereof. Accordingly, for example, the disclosure is also drawn to prodrugs and pharmaceutically acceptable salts of the compositions of the invention, pharmaceutically acceptable salts of such prodrugs, and other bioequivalents.

15 The term "prodrug" indicates a therapeutic agent that is prepared in an inactive form that is converted to an active form (i.e., drug) within the body or cells thereof by the action of endogenous enzymes or other chemicals and/or conditions. In particular, prodrug versions of the oligonucleotides of the invention are prepared as SATE [(S-acetyl-2-thioethyl) phosphate] derivatives according to the methods disclosed in WO 93/24510 to 20 Gosselin *et al.*, published December 9, 1993 or in WO 94/26764 and U.S. 5,770,713 to Imbach *et al.*

The term "pharmaceutically acceptable salts" refers to physiologically and pharmaceutically acceptable salts of the oligomeric compounds of the invention: i.e., salts that retain the desired biological activity of the parent compound and do not impart 25 undesired toxicological effects thereto. For oligonucleotides, preferred examples of pharmaceutically acceptable salts and their uses are further described in U.S. Patent 6,287,860, which is incorporated herein in its entirety.

The present invention also includes pharmaceutical compositions and formulations which include the compositions of the invention. The pharmaceutical compositions of the 30 present invention may be administered in a number of ways depending upon whether local or systemic treatment is desired and upon the area to be treated. Administration may be topical (including ophthalmic and to mucous membranes including vaginal and rectal

delivery), pulmonary, e.g., by inhalation or insufflation of powders or aerosols, including by nebulizer; intratracheal, intranasal, epidermal and transdermal), oral or parenteral.

Parenteral administration includes intravenous, intraarterial, subcutaneous, intraperitoneal or intramuscular injection or infusion; or intracranial, e.g., intrathecal or intraventricular, administration. Oligonucleotides with at least one 2'-O-methoxyethyl modification are believed to be particularly useful for oral administration. Pharmaceutical compositions and formulations for topical administration may include transdermal patches, ointments, lotions, creams, gels, drops, suppositories, sprays, liquids and powders. Conventional pharmaceutical carriers, aqueous, powder or oily bases, thickeners and the like may be necessary or desirable. Coated condoms, gloves and the like may also be useful.

The pharmaceutical formulations of the present invention, which may conveniently be presented in unit dosage form, may be prepared according to conventional techniques well known in the pharmaceutical industry. Such techniques include the step of bringing into association the active ingredients with the pharmaceutical carrier(s) or excipient(s). In general, the formulations are prepared by uniformly and intimately bringing into association the active ingredients with liquid carriers or finely divided solid carriers or both, and then, if necessary, shaping the product.

The compositions of the present invention may be formulated into any of many possible dosage forms such as, but not limited to, tablets, capsules, gel capsules, liquid syrups, soft gels, suppositories, and enemas. The compositions of the present invention may also be formulated as suspensions in aqueous, non-aqueous or mixed media. Aqueous suspensions may further contain substances which increase the viscosity of the suspension including, for example, sodium carboxymethylcellulose, sorbitol and/or dextran. The suspension may also contain stabilizers.

Pharmaceutical compositions of the present invention include, but are not limited to, solutions, emulsions, foams and liposome-containing formulations. The pharmaceutical compositions and formulations of the present invention may comprise one or more penetration enhancers, carriers, excipients or other active or inactive ingredients.

Emulsions are typically heterogenous systems of one liquid dispersed in another in the form of droplets usually exceeding 0.1 μm in diameter. Emulsions may contain additional components in addition to the dispersed phases, and the active drug which may be present as a solution in either the aqueous phase, oily phase or itself as a separate phase.

Microemulsions are included as an embodiment of the present invention. Emulsions and their uses are well known in the art and are further described in U.S. Patent 6,287,860, which is incorporated herein in its entirety.

Formulations of the present invention include liposomal formulations. As used in the present invention, the term "liposome" means a vesicle composed of amphiphilic lipids arranged in a spherical bilayer or bilayers. Liposomes are unilamellar or multilamellar vesicles which have a membrane formed from a lipophilic material and an aqueous interior that contains the composition to be delivered. Cationic liposomes are positively charged liposomes which are believed to interact with negatively charged DNA molecules to form a stable complex. Liposomes that are pH-sensitive or negatively-charged are believed to entrap DNA rather than complex with it. Both cationic and noncationic liposomes have been used to deliver DNA to cells.

Liposomes also include "sterically stabilized" liposomes, a term which, as used herein, refers to liposomes comprising one or more specialized lipids that, when incorporated into liposomes, result in enhanced circulation lifetimes relative to liposomes lacking such specialized lipids. Examples of sterically stabilized liposomes are those in which part of the vesicle-forming lipid portion of the liposome comprises one or more glycolipids or is derivatized with one or more hydrophilic polymers, such as a polyethylene glycol (PEG) moiety. Liposomes and their uses are further described in U.S. Patent 6,287,860, which is incorporated herein in its entirety.

The pharmaceutical formulations and compositions of the present invention may also include surfactants. The use of surfactants in drug products, formulations and in emulsions is well known in the art. Surfactants and their uses are further described in U.S. Patent 6,287,860, which is incorporated herein in its entirety.

In one embodiment, the present invention employs various penetration enhancers to effect the efficient delivery of nucleic acids, particularly oligonucleotides. In addition to aiding the diffusion of non-lipophilic drugs across cell membranes, penetration enhancers also enhance the permeability of lipophilic drugs. Penetration enhancers may be classified as belonging to one of five broad categories, *i.e.*, surfactants, fatty acids, bile salts, chelating agents, and non-chelating non-surfactants. Penetration enhancers and their uses are further described in U.S. Patent 6,287,860, which is incorporated herein in its entirety.

One of skill in the art will recognize that formulations are routinely designed according to their intended use, i.e. route of administration.

Preferred formulations for topical administration include those in which the oligonucleotides of the invention are in admixture with a topical delivery agent such as lipids, liposomes, fatty acids, fatty acid esters, steroids, chelating agents and surfactants. Preferred lipids and liposomes include neutral (e.g. dioleoylphosphatidyl DOPE ethanolamine, dimyristoylphosphatidyl choline DMPC, distearoylphosphatidyl choline) negative (e.g. dimyristoylphosphatidyl glycerol DMPG) and cationic (e.g. dioleoyltetramethylaminopropyl DOTAP and dioleoylphosphatidyl ethanolamine DOTMA).

For topical or other administration, oligonucleotides of the invention may be encapsulated within liposomes or may form complexes thereto, in particular to cationic liposomes. Alternatively, oligonucleotides may be complexed to lipids, in particular to cationic lipids. Preferred fatty acids and esters, pharmaceutically acceptable salts thereof, and their uses are further described in U.S. Patent 6,287,860, which is incorporated herein in its entirety. Topical formulations are described in detail in United States patent application 09/315,298 filed on May 20, 1999, which is incorporated herein by reference in its entirety.

Compositions and formulations for oral administration include powders or granules, microparticulates, nanoparticulates, suspensions or solutions in water or non-aqueous media, capsules, gel capsules, sachets, tablets or minitables. Thickeners, flavoring agents, diluents, emulsifiers, dispersing aids or binders may be desirable. Preferred oral formulations are those in which oligonucleotides of the invention are administered in conjunction with one or more penetration enhancers surfactants and chelators. Preferred surfactants include fatty acids and/or esters or salts thereof, bile acids and/or salts thereof. Preferred bile acids/salts and fatty acids and their uses are further described in U.S. Patent 6,287,860, which is incorporated herein in its entirety. Also preferred are combinations of penetration enhancers, for example, fatty acids/salts in combination with bile acids/salts. A particularly preferred combination is the sodium salt of lauric acid, capric acid and UDCA. Further penetration enhancers include polyoxyethylene-9-lauryl ether, polyoxyethylene-20-cetyl ether. Oligonucleotides of the invention may be delivered orally, in granular form including sprayed dried particles, or

complexed to form micro or nanoparticles. Oligonucleotide complexing agents and their uses are further described in U.S. Patent 6,287,860, which is incorporated herein in its entirety. Oral formulations for oligonucleotides and their preparation are described in detail in United States applications 09/108,673 (filed July 1, 1998), 09/315,298 (filed May 20, 1999) and 10/071,822, filed February 8, 2002, each of which is incorporated herein by reference in their entirety.

Compositions and formulations for parenteral, intrathecal or intraventricular administration may include sterile aqueous solutions which may also contain buffers, diluents and other suitable additives such as, but not limited to, penetration enhancers, carrier compounds and other pharmaceutically acceptable carriers or excipients.

Certain embodiments of the invention provide pharmaceutical compositions containing one or more of the compositions of the invention and one or more other chemotherapeutic agents which function by a non-antisense mechanism. Examples of such chemotherapeutic agents include but are not limited to cancer chemotherapeutic drugs such as daunorubicin, daunomycin, dactinomycin, doxorubicin, epirubicin, idarubicin, esorubicin, bleomycin, mafosfamide, ifosfamide, cytosine arabinoside, bis-chloroethylnitrosurea, busulfan, mitomycin C, actinomycin D, mithramycin, prednisone, hydroxyprogesterone, testosterone, tamoxifen, dacarbazine, procarbazine, hexamethylmelamine, pentamethylmelamine, mitoxantrone, amsacrine, chlorambucil, methylcyclohexylnitrosurea, nitrogen mustards, melphalan, cyclophosphamide, 6-mercaptopurine, 6-thioguanine, cytarabine, 5-azacytidine, hydroxyurea, deoxycoformycin, 4-hydroxyperoxycyclophosphoramide, 5-fluorouracil (5-FU), 5-fluorodeoxyuridine (5-FUdR), methotrexate (MTX), colchicine, taxol, vincristine, vinblastine, etoposide (VP-16), trimetrexate, irinotecan, topotecan, gemcitabine, teniposide, cisplatin and diethylstilbestrol (DES). When used with the compositions of the invention, such chemotherapeutic agents may be used individually (*e.g.*, 5-FU and oligonucleotide), sequentially (*e.g.*, 5-FU and oligonucleotide for a period of time followed by MTX and oligonucleotide), or in combination with one or more other such chemotherapeutic agents (*e.g.*, 5-FU, MTX and oligonucleotide, or 5-FU, radiotherapy and oligonucleotide). Anti-inflammatory drugs, including but not limited to nonsteroidal anti-inflammatory drugs and corticosteroids, and antiviral drugs, including but not limited to ribivirin, vidarabine, acyclovir and ganciclovir, may also be combined in compositions of the invention.

Combinations of compositions of the invention and other non-antisense drugs are also within the scope of this invention. One or more compositions of the invention can be used in combination with other therapeutic agents to create a cocktail as is currently the strategy for certain viral infections.

5 In another related embodiment, therapeutically effective combination therapies may comprise the use of two or more compositions of the invention wherein the multiple compositions are targeted to a single or multiple nucleic acid targets. Numerous examples of antisense oligomeric compounds are known in the art. Two or more combined compounds may be used together or sequentially

10

Dosing

The formulation of therapeutic compositions and their subsequent administration (dosing) is believed to be within the skill of those in the art. Dosing is dependent on severity and responsiveness of the disease state to be treated, with the course of treatment

15 lasting from several days to several months, or until a cure is effected or a diminution of the disease state is achieved. Optimal dosing schedules can be calculated from measurements of drug accumulation in the body of the patient. Persons of ordinary skill can easily determine optimum dosages, dosing methodologies and repetition rates. Optimum dosages may vary depending on the relative potency of individual

20 oligonucleotides, and can generally be estimated based on EC₅₀s found to be effective in *in vitro* and *in vivo* animal models. In general, dosage is from 0.01 ug to 100 g per kg of body weight, and may be given once or more daily, weekly, monthly or yearly, or even once every 2 to 20 years. Persons of ordinary skill in the art can easily estimate repetition rates for dosing based on measured residence times and concentrations of the drug in

25 bodily fluids or tissues. Following successful treatment, it may be desirable to have the patient undergo maintenance therapy to prevent the recurrence of the disease state, wherein the oligonucleotide is administered in maintenance doses, ranging from 0.01 ug to 100 g per kg of body weight, once or more daily, to once every 20 years.

30 While the present invention has been described with specificity in accordance with certain of its preferred embodiments, the following examples serve only to illustrate the invention and are not intended to limit the same.

Example 1

Preparation of Human RNase H1.

Human RNase H1 containing an N-terminal His-tag was expressed and purified as previously described (28). Briefly, the plasmids were transfected into *E. coli* BL21 (DE3) (Novagen, WI). The bacteria was grown in Terrific Broth medium (Boi 101 Systems) at 37° C and harvested at OD₆₀₀ of 1.2. The cells were induced with 1 mM isopropylthiogalactoside (IPTG) at 37° C for 2 h. The cells were lysed in 6 M guanidine hydrochloride 100 mM sodium phosphate, 10 mM tris, pH 8.0 for 16 – 20 h at 24° C. The recombinant proteins were incubated for 1 h with 1 mL of Ni-NTA Super flow beads (Qiagen) per 50 mL of lysate. The Ni-NTA media was packed into an FPLC column and the RNase H1 proteins partially purified with sequential gradients (flow rate, 5 mL/min; buffer A, 100 mM sodium phosphate, 10 mM tris-HCl, 8 M Urea, pH 6.3; buffer B, 100 mM sodium phosphate, 10 mM tris-HCl, 2 M Urea, pH 6.3; buffer C, 100 mM sodium phosphate, 10 mM tris-HCl, 2 M Urea, 100 mM EDTA, pH 7.0). The eluent was further purified by ion exchange FPLC chromatography (Mono S Column; flow rate, 1 mL/min; buffer A, 20 mM sodium phosphate, 2 M urea, 200 mM NaCl, pH 7.0; buffer B, 20 mM sodium phosphate, 2 M urea, 2 M NaCl, pH 7.0). Fractions containing RNase H1 were pooled and concentrated. The concentrated protein was purified by RP-FPLC (Resource RPC Column; flow rate 1 mL/min; Buffer A, 2% acetonitrile in diH₂O, 0.065% trifluoroacetic acid; buffer B 80% acetonitrile in diH₂O, 0.05% trifluoroacetic acid). Fractions were lyophilized, resuspended in diH₂O and analyzed by SDS-PAGE.

Synthesis of oligonucleotides

The oligoribonucleotides were synthesized on a PE-ABI 380B synthesizer using 5'-O-silyl-2'-O-bis(2-acetoxyethoxy)methyl ribonucleoside phosphoramidites and procedures described elsewhere (29). The oligoribonucleotides were purified by reverse-phase HPLC. The oligodeoxyribonucleotides were synthesized on a PE-ABI 380B automated DNA synthesizer and standard phosphoramidite chemistry. The oligodeoxyribonucleotides were purified by precipitation 2 times out of 0.5 M NaCl with 2.5 volumes of ethyl alcohol.

Synthesis of modified oligonucleotides

The 1,4-anhydro-5-*O*-(4,4'-dimethoxytrityl)-2-deoxy-D-erythro-pentenol -3-[(2-cyanoethyl)-*N,N*-diisopropyl]phosphoramidite, 5'-*O*-(4,4'-dimethoxytrityl)-3-(4-methylbenzoyl)-2-thio-thymidine-3'-[(2-cyanoethyl)-*N,N*-diisopropyl]phosphoramidite, 5'-*O*-(4,4'-dimethoxytrityl)-3'-deoxypseudouridine-3'-[(2-cyanoethyl)-*N,N*-diisopropyl]phosphoramidite, 1',2'-dideoxy-1'-(2,4-difluorotoluy l)-5'-*O*-(4,4'-dimethoxytrityl)- β -D-ribofuranose-3'-[(2-cyanoethyl)-*N,N*-diisopropyl]phosphoramidite and 5'-*O*-(4,4'-dimethoxytrityl)-3-(4-methylbenzoyl)-2'-uridine-3'-[(2-cyanoethyl)-*N,N*-diisopropyl]phosphoramidite were procured from commercial sources (Glen Reseach Inc., Virginia, U. S. A). The 1-[2-deoxy-2-fluoro-5-*O*-(4,4'-dimethoxytrityl)-3-[(2-cyanoethyl)-*N,N*-diisopropyl]phosphoramidite- β -D-[arabinofur anosyl]-thymine, 5'-*O*-(4,4'-dimethoxytrityl)-2'-deoxy-2'-fluoro-thymidine-3'-[(2-cyanoethyl)-*N,N*-diisopropyl]phosphoramidite, 2-fluoro-6-methylbenzoimidazole deoxyribonucleotide, 4-methylbenzoimidazole deoxyribonucleotide, hydrocarbon linkers and 5'-*O*-(4,4'-dimethoxytrityl)-2'-S-methyl-2'-thio-5-methyluridine-3'-[(2-cyanoethyl)-*N,N*-diisopropyl]phosphoramidite were synthesized as reported (30-32). The nucleoside 3'- \square -C-methylthymidine was synthesized from 1,2-*O*-isopropylidene-D-xylofuranose and converted to the 5'-*O*-(4,4'-dimethoxytrityl)-3'-*O*-(2-cyanoethyl-diisopropylamino)-phosphoramidite as previously described (33). The 4'- \square -C-methylthymidine nucleoside was synthesized in 12 steps (manuscript in preparation) starting from commercially available 1,2:5,6-Di-*O*-isopropylidene- \square -D-glucofuranose purchased from Pfanstiehl, Waukegan, IL. The alternate synthesis of this nucleoside has been reported (34-35). It was converted to 5'-*O*-(4,4'-dimethoxytrityl)-4'- \square -C-methylthymidine-3'-*O*-(2-cyanoethyl)-*N,N*-Diisopropylphosphoramidite by following the procedure described for similar compounds (36-37). Standard phosphoramidites and solid supports were used for incorporation of A, T, G, and C residues. A 0.1 M solution of each amidite in anhydrous acetonitrile was used for the synthesis of modified oligonucleotides. The oligonucleotides were synthesized on functionalized controlled pore glass (CPG) on an automated solid phase DNA synthesizer with the final DMT group retained at 5'-end. For incorporation of modified amidites, 6 equivalent of phosphoramidite solutions were delivered in two portions, each followed by a 3 min coupling wait time. All other steps in the protocol supplied by the manufacturer were used without modification. Oxidation of the internucleotide phosphite to the phosphate was carried out using 0.1 M solution of iodine

in pyridine/water (20/1, v/v). with 10 min oxidation wait time. The coupling efficiencies were more than 97%. To deprotect oligonucleotides containing 2'-deoxy-2'-fluorothymidine and 2'-deoxy-2'-fluoroarabinofuranosylthymine, the solid support bearing the oligonucleotides were suspended in aqueous ammonia (28-30 wt%):ethanol (3:1, 3 mL for 5 2 μ mol scale synthesis) and heated at 55 °C for 6 h. All other modified oligonucleotides after completion of the synthesis, the solid supports bearing the oligonucleotides were suspended in aqueous ammonium hydroxide (28-30 wt %, 2 mL for 2 μ mol scale synthesis) and kept at room temperature for 2 h. The solid support was filtered and the filtrate was heated at 55 °C for 6 h to complete the removal of all protecting groups. Crude 10 oligonucleotides were purified by high performance liquid chromatography (HPLC, C-4 column, Waters, 7.8 x 300 mm, A = 100 mM ammonium acetate, pH 6.5-7, B = acetonitrile, 5-60% of B in 55 min, flow 2.5 mL min⁻¹, λ 260 nm). Detritylation was achieved by adjusting the pH of the solution to 3.8 with acetic acid and keeping at room temperature until complete removal of the trityl group, as monitored by HPLC analysis.

15 The oligonucleotides were then desalted by HPLC to yield modified oligonucleotides in 30-40% isolated yield calculated based on the loading of the 3'-base to solid support (38). The oligonucleotides were characterized by electrospray mass spectroscopy (ES-MS) and their purity was assessed by HPLC and capillary gel electrophoresis (CGE). The purity of the oligonucleotides was > 90%.

20 Oligonucleotides with abasic sites were conveniently generated by the use of uracil-DNA glycosylase (39). Oligonucleotides containing deoxyuridine residue were synthesized as described (vide supra). The HPLC purified oligonucleotides (0.32 mg) were dissolved in Uracil-DNA Glycosylase (149 μ l, 1 unit in 1 μ l dissolved in 30 mM HEPES-KOH (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM DTT, 0.05% Tween 20 and 25 50% glycerol) and incubated at 37 °C for 4 h. The reaction was terminated by filtering the enzyme using low binding membrane filter (0.22 μ m, Millipore Inc., Bedford, MA, USA). The release of uracil was observed in HPLC analysis of the reaction mixture (Waters C-4 3.9 x 300 mm, , delta pack, 15 micron, 300 A°, A = 100mM ammonium acetate, B = acetonitrile 0 to 25 % B in 55 min, flow 1.5 ml min⁻¹, λ 260 nm) and co injecting the 30 authentic sample. The oligonucleotides were purified by HPLC (conditions same above). The purity (> 90%) of the oligonucleotides was assed by HPLC analysis.

Preparation of ^{32}P labeled substrate

The RNA substrate was 5'-end-labeled with ^{32}P using 20 U of T4 polynucleotide kinase (Promega, WI), 120 pmol (7000 Ci/mmol) [$\gamma\text{-}^{32}\text{P}$]ATP (ICN, CA), 40 pmol RNA, 70 mM tris, pH 7.6, 10 mM MgCl_2 and 50 mM DTT. The kinase reaction was incubated at 37° C for 30 min. The labeled oligoribonucleotide was purified by electrophoresis on a 12% denaturing polyacrylamide gel (40). The specific activity of the labeled oligonucleotide is approximately 3000 to 8000 cpm/fmol.

10 *Preparation of the heteroduplex*

The heteroduplex substrate was prepared in 100 μL containing unlabeled oligoribonucleotide ranging from 100 to 1000 nM, 10^5 cpm of ^{32}P labeled oligoribonucleotide, two-fold excess complementary oligodeoxyribonucleotide and hybridization buffer [20 mM tris, pH 7.5, 20 mM KCl]. Reactions were heated at 90° C for 5 min, cooled to 37° C and 60 U of Prime RNase Inhibitor (5 Prime \rightarrow 3 Prime, CO) and MgCl_2 at a final concentration of 1 mM were added. Hybridization reactions were incubated 2 - 16 h at 37° C and 1 mM tris(2-carboxyethyl)phosphate (TCEP) was added.

Multiple-turnover kinetics

20 The human RNase H1 proteins were incubated with dilution buffer (50 mM tris, 50 mM NaCl, 100 μM TCEP, pH 7.5) for 1 h at 24° C. The heteroduplex substrate was digested with 0.4 ng of enzyme at 37° C. A 10 μL aliquot of the cleavage reaction was removed at time points ranging from 2 - 120 min and quenched by adding 5 μL of stop solution (8 M urea and 120 mM EDTA). The aliquots were heated at 90° C for 2 min, resolved in a 12% denaturing polyacrylamide gel and the substrate and product bands were quantitated on a Molecular Dynamics PhosphorImager. The concentration of the converted product was plotted as a function of time. The initial cleavage rate (V_0) was obtained from the slope (mole RNA cleaved/min) of the best-fit line for the linear portion of the plot, which comprises, in general < 10% of the total reaction and data from at least five time points.

30 Site-specific cleavage rates were determined by plotting the concentration of the converted product for a given cleavage site as a function of time.

Results

Oligodeoxyribonucleotides were prepared with nucleotide modifications positioned within the region preferentially cleaved by human RNase H1 (Fig. 1C). The deoxyribonucleotide modifications consisted of sugar modifications (e.g., 2'-methylthiothymidine, 2'-ara-fluoropyrimidine, 4'-methylthymidine, 3'-methylthymidine, cyclohexenylthymidine and 2'-fluorothymidine) as well as base modifications (e.g., 2-thiouridine, pseudouridine, 2-fluoro-6-methylbenzoimidazole deoxyribonucleotide, 4-methylbenzoimidazole deoxyribonucleotide and 2,4-difluorotoluidyl deoxyribonucleotide) (Fig. 1A). In addition, the deoxyribonucleotides were substituted with hydrocarbon linkers ranging in length from 3 to 5 residues, ganciclovir, tetrahydrofuran and abasic deoxyribonucleotide (Fig. 1B). The modified oligodeoxyribonucleotides were annealed to complementary RNA and the heteroduplexes digested with human RNase H1 under multiple-turnover conditions as described in Materials and Methods. Initial cleavage rates (V_0) as well as site-specific cleavage rates, i.e., initial cleavage rates for each human RNase H1 cleavage site, were determined (Table I).

Figure 2 shows examples of the human RNase H1 cleavage patterns and site-specific cleavage rates for the unmodified substrate and heteroduplexes containing the 4'-methylthymidine at position T_9 and 2-thiouridine at position T_8 . The 2-thiouridine modified deoxyribonucleotide dramatically reduced the site-specific cleavage rates for the ribonucleotide opposing the modified nucleotide and the adjacent 3'-ribonucleotide, i.e., positions 0 and +1, resulting in a 30 percent reduction in the V_0 observed for this heteroduplex (Fig. 2 and Table 1A). The 4'-methylthymidine modification exhibited a significantly greater effect on human RNase H1 activity. In this case, no human RNase H1 cleavage was observed for the ribonucleotide opposing the 4'-methylthymidine and the two adjacent 3' and two adjacent 5'-ribonucleotides, i.e., positions -2 to +3 (Fig. 2 and Table 1A). In addition, all the site-specific cleavage rates for the heteroduplex containing the 4'-methylthymidine were slower when compared to the unmodified substrate, resulting in a 3-fold reduction in the initial cleavage rate (Table I). These data suggest that the degrees to which the initial cleavage rates were reduced for the modified heteroduplexes were a function of the number of human RNase H1 cleavage sites either reduced or ablated (Table I). The effects did not vary as a function of the specific nucleotide

modified. For example, site-specific cleavage rates at the ribonucleotide opposing an abasic modification was 20, 50 and 20 percent of the unmodified substrate (Table 1B).

The initial cleavage rates (V_0) observed for the modified heteroduplexes were predominantly dependant on the class of modification tested rather the position of the specific modification within the oligodeoxynucleotide (Table I). For example, several modified heteroduplexes (e.g., 4'-methylthymidine, tetrahydrofuran, gancyclovir and the hydrocarbon linkers) exhibited initial cleavage rates 2 to 3-fold slower than the V_0 of the unmodified substrate whereas the 2'-ara-fluoropyrimidine, pseudouridine and Π -stacking deoxyribonucleotides, (e.g., 2-fluoro-6-methylbenzoimidazole, 4-methylbenzoimidazole and 2,4-difluorotoluy deoxyribonucleotides) modified heteroduplexes exhibited initial cleavage rates comparable to the rate observed for the unmodified substrate (Table I). It is important to note that a 2-fold reduction of the initial cleavage rate due to a single nucleotide modification is significant considering that human RNase H1 cleaves the substrate at multiple positions within the heteroduplex. In contrast, the initial cleavage rates for the heteroduplexes containing the same modification at different positions within the substrate showed only a ± 10 percent difference in the cleavage rates (Table I). Nor did the effects vary as a function of the specific nucleotide modified. For example, the cleavage rates at the ribonucleotide opposing an abasic site was 0.2, 0.5 and 0.2 of the control at, respectively, positions 9, 10 and 11. The relative cleavage rates at positions 9 and 11 were comparable even though positions 9 and 11 in the natural substrates were thymidine and cytosine. Moreover, the effects on the cleavage at adjacent sites were comparable. Similar results were observed for other modifications that produced dramatic reductions in the cleavage rates, (e.g., 2'-fluoro) and modifications that had little to no effect on the cleavage rates, (2'-ara-fluoro).

The modifications that exhibited the greatest impact on the site-specific cleavage rate for the ribonucleotide opposing the modification also exhibited the broadest effect on the site-specific cleavage rates surrounding ribonucleotides. The tetrahydrofuran, hydrocarbon linkers, 4'-methylthymidine and abasic deoxyribonucleotide modifications which significantly reduced or ablated the site-specific cleavage rates for the ribonucleotide opposing the modification also showed significantly slower site-specific cleavage rates for the surrounding 3' and 5'-ribonucleotides, (e.g., positions -2 to +2) compared with the unmodified substrate (Table I). Interestingly, with the exception of 4'-

methylthymidine, these modifications were predicted to impart the greatest degree of conformational flexibility at the site of the modification. The heteroduplexes containing the pseudouridine, 2'-ara-fluoropyrimidine, 3'-methylthymidine and Π -stacking deoxyribonucleotides modifications which exhibited little to no reduction in the site-specific cleavage rate for the ribonucleotide opposing the modification also showed only a modest reduction in the site-specific rates for the surrounding ribonucleotides (Table I). For a majority of the modified deoxyribonucleotides tested, the influence on the human RNase H1 activity of the adjacent ribonucleotides appeared to be unidirectional (Fig. 1A). For example, the 2'-methylthiothymidine, 3'-methylthymidine, 2-thiouridine, 2'-fluorothymidine and pseudouridine reduced the site-specific cleavage rates for the adjacent 3'-ribonucleotides more significantly than the 5'-ribonucleotides.

Although there was generally quite a good correlation between the effects on the cleavage rate at the ribonucleotide opposing the modification and the overall cleavage rate, there were interesting exceptions. Consider 2'-thiothymidine, this modification at position 8 ablated the cleavage at the opposing site and reduced the relative overall rate to 0.7. While the same modification at position 7 reduced the relative site-specific rate to 0.2 of control and the overall relative rate to 0.5. These results can be explained different effects on the site-specific cleavage rates of adjacent ribonucleotides. In contrast, the effects of the 4'-methylthymidine modifications on the overall rates were less significant than on the site-specific rates for the opposing and surrounding ribonucleotides and this is due to the fact that the ablated cleavage sites account for approximately half of the total site-specific cleavage rates. These results emphasize the importance of performing both overall and site-specific measurements.

Within the context of nucleic acid duplexes, the degree of pseudorotation of the sugar between the southern to the northern conformation sets into motion a series of structural changes that ultimately result in the formation of B-form or A-form duplexes (25). The shift in helical conformation resulting from the pseudorotation of the sugar starts with the change in the torsion angles of the glycosyl and C4-C5 bonds. The spatial orientation of the glycosyl bond in turn dictates the distance between the internucleotide phosphates, the rotation and axial rise per nucleotide, the tilt of the base-pair and the

dislocation of the base pairs from the helical axis. These factors combined, control the depth and width of the major and minor grooves (for review see: 25).

RNA/DNA heteroduplexes are unique in that these duplexes exhibit an intermediate structure between the canonical A-form and B-form helical geometry. At approximately 9Å the minor groove width for the heteroduplex is approximately midway between the minor groove widths for A-form ($\approx 11\text{Å}$) and B-form ($\approx 6\text{Å}$) duplexes (23-25). The RNA sugars of the heteroduplex exhibit a northern sugar conformation whereas the DNA sugars form an eastern O_4' -endo sugar conformation. The intrastrand phosphate distances for the heteroduplex also differ from canonical A-form and B-form duplexes in that the DNA strand maintains an intrastrand phosphate distance consistent with B-form duplexes whereas the intrastrand phosphate distance within the RNA strand is closer to an A-form duplex. Finally, the unique minor groove width as well as the position of the inter- and intrastrand phosphates exhibited by the DNA/RNA heteroduplexes suggest that these features may be the key recognition determinants for RNase H enzymes.

Although, the key catalytic amino acids of human RNase H1 have been identified, the structural and physical properties of the enzyme and substrate responsible for the selective recognition and cleavage of the RNA in the RNA/DNA heteroduplex are not known as a cocrystal structure of the enzyme/substrate complex has not been solved (13). Site-directed mutagenesis of the *E. coli* and human RNase H1 enzymes combined with molecular modeling of the enzyme/substrate complex suggest that the enzyme binds to the minor groove of the heteroduplex substrate (13, 41-42, Lima unpublished). In addition, the catalytic site of the enzyme was predicted to contact the 2'-hydroxyls of the RNA strand and the phosphates of the DNA strand surrounding the scissile phosphate (Fig. 3).

In this study we performed a complementary mutational analysis on the structure of the substrate at the catalytic site for human RNase H1. We designed a series of modified heteroduplexes with the modifications positioned within the catalytic site of the substrate (Fig. 1). The modifications consisted of nucleotides exhibiting northern, southern and eastern sugar conformations, base modifications which Π -stack with adjacent nucleotides but do not form hydrogen bonds, abasic deoxynucleotides, internucleotide hydrocarbon linkers ranging 3-5 residues and ganciclovir substitution of the deoxyribose to determine the role of helical geometry, sugar conformation, bulk in the minor groove and conformational flexibility within the heteroduplex on human RNase H1 activity.

The role of sugar conformation within the catalytic site of the heteroduplex

The northern biased deoxyribonucleotides selected for this study included the 2'-fluorothymidine and 2-thiouridine modifications (Fig. 1A). All three modifications lacked bulky 2'-substituents in order to avoid possible steric interactions with the enzyme. These modifications were determined to influence sugar conformation through distinctly different mechanisms. For example, the highly electronegative fluorine of the 2'-fluorothymidine acts in conjunction with the gauche effect to strongly stabilize the sugar in the northern pucker (30). In the case of 2-thiouridine, it has been shown at the dinucleotide level that the highly polarizable sulfur stabilizes the C_{3'}-*endo* sugar conformation as well as the stacking interactions with the neighboring nucleotides and imparts stronger hydrogen bonding due to the increased acidity of the N-3 imino proton (43-45). The heteroduplexes containing the northern biased modifications showed significantly slower site-specific cleavage rates for the ribonucleotide opposing the modification (position 0) (Table IA). Further, these modified heteroduplexes also exhibited significantly slower site-specific cleavage rates for the adjacent 3'-ribonucleotide, i.e., position +1, while little to no reduction for the site-specific cleavage rates was observed for the adjacent 5'-ribonucleotide, i.e., position -1, thus these modifications appear to be influencing the structure of the adjacent base-pairs in a unidirectional manner (Table IA).

The role of the minor groove substituents

In contrast to northern biased deoxyribonucleotides, modified deoxyribonucleotides exhibiting a southern biased sugar pucker more closely mimic the sugar conformation of native deoxyribonucleotides. Surprisingly, the effects of southern biased deoxyribonucleotide modifications on RNase H1 activity have not been previously investigated. Heteroduplexes containing the 2'-methylthiothymidine modification were poor substrates for human RNase H1 exhibiting significantly slower initial cleavage rates (V_0) and site-specific cleavage rates for both the opposing and adjacent ribonucleotides (Table IA). The 2'-methylthiothymidine nucleoside is highly southern biased as a result of the electronegativity and steric bulk of the 2'-substituent. The 2'-methylthio substituent potentially poses a similar steric problem for the enzyme as the 2'-alkoxy moieties and

may account for the observed loss in human RNase H1 activity. In contrast, the pseudouridine modification had little to no effect on cleavage rates. These data suggest that, consistent with the predicted binding model, human RNase H1 is likely interacting with the minor groove of the heteroduplex substrate and that a low energy barrier for
5 interconversion of the sugar conformation is preferred.

The role of the deoxyribonucleotide phosphate groups

Heteroduplexes containing the 4'-methylthymidine modifications were also poor substrates for human RNase H1 exhibiting initial cleavage rates 2 to 3-fold slower than the
10 unmodified heteroduplex (Table IA). The 4'-methylthymidine inhibited the human RNase H1 cleavage of the ribonucleotide opposing the modification and the adjacent 3'-ribonucleotides. The site-specific rates for the adjacent 5'-ribonucleotides were significantly reduced compared to the unmodified substrate. In contrast, the heteroduplexes containing the 3'-methyl modified deoxyribonucleotide, which have been
15 shown to exhibit a sugar conformation similar to the 4'-methyl nucleosides, were significantly better substrates for human RNase H1. The observed differences in the human RNase H1 activity for the 4'-methylthymidine and 3'-methylthymidine heteroduplexes may be a function of the position of the 4'-methyl moiety on the furanose ring, which is predicted to contribute bulk in the minor groove potentially interfering with
20 enzyme binding (37). Furthermore, the loss in human RNase H1 activity observed for the 4'-methylthymidine heteroduplexes suggests that proper orientation of the phosphate group on the deoxyribonucleotide opposing the scissile ribonucleotide is important for human RNase H1 activity.

25 Optimal modification mimic the conformation of deoxyribonucleotides

The nucleotide modifications predicted to mimic the sugar pucker of the deoxyribonucleotide of an RNA/DNA heteroduplex, (e.g., heteroduplexes containing the 2'-ara-fluoropyrimidines and pseudouridine modifications) exhibited cleavage rates comparable to the rates observed for the unmodified substrate (Table IA). The 2'-ara-
30 fluoro modification has been shown by NMR to form the eastern *O₄-endo* sugar conformation similar to DNA when hybridized to RNA (26). In addition, the size and position of the 2'-ara-substituent, i.e., the fluorine is directed upward and away from the

minor groove, is predicted not to sterically interfere with the enzyme. In the case of the pseudouridine, the NMR structure indicated a modestly higher southern sugar pucker population for the nucleoside due to the influence of the torsion angle of the C-C glycosyl bond and the C_{1'} and C_{2'} bond (46). Furthermore, the CD spectrum for

5 RNA/pseudouridine and DNA/pseudouridine duplexes showed, respectively, an RNA-like C_{3'}-*endo* and DNA-like C_{2'}-*endo* sugar pucker for the pseudouridine deoxyribonucleotides suggesting that pseudouridine exhibits a conformational flexibility comparable to DNA (47-48). Apparently, both the eastern sugar pucker and conformational flexibility of the deoxyribonucleotide furanose ring are favored by the enzyme, i.e., locking the sugar north

10 or south resulted in slower cleavage rates.

Role of conformational flexibility within the catalytic site of the heteroduplex

Conformational flexibility was introduced at the catalytic site of the heteroduplex substrate with modifications exhibiting incrementally increasing flexibility with the

15 hydrocarbon linkers predicted to exhibit the greatest degree of conformational flexibility followed by abasic and ganciclovir deoxyribonucleotides and the Π stacking deoxyribonucleotides, (e.g., 2-fluoro-6-methylbenzoimidazole, 4-methylbenzoimidazole and 2,4-difluorotoluy l deoxyribonucleotides) (Fig. 1B). Table IB shows that with increased conformational flexibility at the catalytic site, the initial cleavage rates (V_0) and

20 site-specific cleavage rates decreased. For example, the hydrocarbon linkers were predicted to exhibit the greatest degree of conformational flexibility and were among the poorest substrates for RNase H1 activity. The site-specific rates for the ribonucleotide opposing these modification and the surrounding 3' and 5'-ribonucleotides were either significantly reduced or ablated resulting in initial cleavage rates (V_0) approximately two-

25 fold slower than the unmodified substrate (Table I). The broad effect on the site-specific rates for the ribonucleotides surrounding the opposing ribonucleotide is likely due to the fact that the conformationally flexible linkers bridge both the 3' and 5'-deoxyribonucleotides. Interestingly, similar effects on the cleavage rates were observed for all three hydrocarbon linkers even through the linkers ranged in length from three to

30 five carbons with the propyl and pentyl linkers predicted to be, respectively, shorter and longer than the length of the native deoxyribonucleotide linkage and the butyl linker predicted to most closely approximate the intraphosphate distance of the

deoxyribonucleotide. The ganciclovir, abasic and tetrahydrofuran modified deoxyribonucleotides were also poor substrates for human RNase H1, although the site-specific cleavage rates for these heteroduplexes were slightly faster than the rates observed for the heteroduplexes containing the hydrocarbon linkers (Table IB). Taken together
5 these data suggest that a conformationally rigid phosphate backbone is required for human RNase H1 activity. Furthermore, the slight improvement in the cleavage rates observed for the ganciclovir and abasic modifications compared with the hydrocarbon linkers suggests that the furanose ring of the abasic deoxyribonucleotide and hydrogen bond base-pair formation of the ganciclovir modification likely offer modest conformational rigidity to
10 the substrate.

In contrast, the Π -stacking deoxyribonucleotides, (e.g., 2-fluoro-6-methylbenzoimidazole, 4-methylbenzoimidazole and 2,4-difluorotoluy
deoxyribonucleotides) better supported human RNase H1 activity (Table IB). Comparable initial cleavage rates and site-specific cleavage rates were observed for these
15 heteroduplexes compared to the unmodified substrate. Interestingly, the site-specific cleavage rates for the second 3'-ribonucleotide were significantly slower suggesting that a stable hydrogen bond base-pair is required two base-pairs 5' to the scissile phosphate. The fact that the Π -stacking deoxyribonucleotides exhibited comparable site-specific cleavage rates for the ribonucleotide opposing the modification compared to the unmodified
20 substrate suggests that these modifications likely form favorable stacking interactions resulting in a stable helical conformation. In fact, previous studies have shown that the 2-fluoro-6-methylbenzoimidazole deoxyribonucleotide was an effective substitute of native deoxyribonucleotides and this modification was shown to act as an efficient template for replicating DNA with KF(exo)-polymerase (49).

25

Interactions between enzyme and substrate at the catalytic site

The interactions between RNase H1 and the heteroduplex substrate at the catalytic site has been inferred by molecular modeling and site-directed mutagenesis of *E. coli* and human RNase H1 as well as the crystal structure of the *E. coli* enzyme (Fig. 3) (41).
30 Specifically, site-directed mutagenesis suggests that the glutamine at position 72 of the *E. coli* enzyme forms a hydrogen bond with the 2'-hydroxyl of the ribonucleotide at position -1 and that the backbone imino and carbonyl groups of cysteine-13 were function as

proton donor and acceptor, respectively, in the hydrogen bonding interaction with the 2'-hydroxyl of the ribonucleotide at position +1 (41). The asparagine-16 and asparagine-44 residues of the enzyme were suggested to bind electrostatically with the phosphates of, respectively, the deoxyribonucleotides opposing the scissile ribonucleotide and the
5 ribonucleotide at position -1. The aspartic acid residues at positions 10 and 70 were predicted to bind the 2'-hydroxyl of the scissile ribonucleotide via Mg^{2+} ion coordination (41). Finally, these amino acid residues are conserved in human RNase H1 and have been shown by site-directed mutagenesis to be required for activity (13, 42, Lima unpublished).

The loss of human RNase H1 activity observed for the heteroduplexes containing
10 southern biased 2'-methylthiothymidine modification as well as the lack of cleavage observed for the northern biased 2'-alkoxy modified heteroduplexes is consistent with the predicted binding site for the enzyme (21-22, 27). Together, these data suggest that, irrespective of sugar conformation, bulky 2'-substituents positioned in the minor groove of the heteroduplex substrate interfere with human RNase H1 cleavage. Similarly, the 2-thio
15 substitution of 2-thiouridine is predicted to be situated within the minor groove of the heteroduplex and the slower cleavage rates observed for these heteroduplexes may be the result of the sulfur either interfering with the enzyme sterically or as a result of its strong electronegative properties.

The modified heteroduplexes examined here suggest that the width of the minor
20 groove of the heteroduplex substrate is important for RNase H1 catalysis and that variations in minor groove width as a function of sugar pucker appear to obviate the proper positioning of the enzyme on the heteroduplex substrate. For example, the heteroduplexes containing the 2'-ara-flouro deoxyribonucleotides, which produce a minor groove width comparable to the RNA/DNA heteroduplex, exhibited comparable human
25 RNase H1 cleavage rates. On the other hand, deoxyribonucleotide modifications exhibiting a northern biased sugar conformation, (e.g., 2'-thiothymidine) reduced human RNase H1 activity. Consequently, the wider minor groove generated by these modifications likely precludes the associated metal ion coordination of the enzyme with the 2'-hydroxyl of the scissile ribonucleotide and electrostatic interaction with the
30 phosphate of the modified deoxyribonucleotide (Fig. 3) (41). Similarly, a wider minor groove could account for the observed reduction in the site-specific rates for the adjacent 3'-ribonucleotide by preventing the formation of the putative hydrogen bond between the

enzyme and the 2'-hydroxyl of the ribonucleotide at position +1 and the electrostatic interaction with the phosphate of the opposing deoxyribonucleotide (Fig.3). Consistent with these observations and the proposed model for the interaction of the enzyme with the heteroduplex substrate at the catalytic site, the significantly slower cleavage rates observed for the 4'-methylthymidine heteroduplexes suggest that proper positioning of the deoxyribonucleotide phosphate opposing the scissile ribonucleotide is critical for human RNase H1 cleavage. In addition, these observations suggest that the human RNase H1 activity associated with the deoxyribonucleotides exhibiting the northern versus southern biased sugar conformations is likely the result of differences in the relative positions of the inter- and intranucleotide phosphates on the heteroduplex substrate. Lastly, the cleavage rates observed for the 3'-methylthymidine modified heteroduplexes suggest minor groove widths that are narrower than the RNA/DNA heteroduplex are tolerated better than are wider minor grooves by human RNase H1.

Conformational flexibility of the deoxyribose also appears to be an important structural feature of the heteroduplex substrate for human RNase H1 activity. The preferred eastern *O*_{4'}-*endo* sugar pucker observed for the DNA strand of the heteroduplex is the result of the nearly symmetrical potential energy barrier for both south and north sugar conformations exhibited by deoxyribonucleotides. Both the pseudouridine and 2'-arafluoro-deoxyribonucleotides exhibit conformational flexibility in the sugar and the heteroduplexes containing these modifications showed cleavage rates comparable to the unmodified substrate. Furthermore, modifications exhibiting strong conformationally biased sugars, (e.g., 2'-fluoro-deoxyribonucleotides) were less efficiently cleaved by the enzyme.

Whereas conformational flexibility of the deoxyribose was preferred, flexibility in the in the phosphate backbone of the DNA strand inhibited human RNase H1 activity. Modifications such as the hydrocarbon linkers and abasic deoxyribonucleotides that permit free rotation of the phosphate moiety were shown to inhibit human RNase H1 activity. Again these data suggest that proper positioning the phosphate groups of the deoxyribonucleotide, presumably for electrostatic contact with the enzyme, is essential for human RNase H1 catalysis. The cleavage rates observed for the Π -stacking deoxyribonucleotides suggest that stable base-stacking independent of hydrogen bond formation between the bases at the catalytic site appeared to offer sufficient rigidity to the

phosphate backbone. Taken together these data suggest that variation in sugar conformation is significantly better tolerated by human RNase H1 than conformational flexibility in the phosphate backbone.

Previous studies have shown that nucleotides exhibiting conformationally biased
5 sugars can bias the sugar conformation of the surrounding deoxyribonucleotides. For example, the NMR structures of chimeric RNA-DNA/RNA heteroduplexes show that the deoxyribonucleotides adjacent to the RNA of the chimeric strand adopts the northern pucker of the RNA (50). The transmission the northern sugar conformation of the RNA into the adjacent deoxyribonucleotides is likely due the intrinsically flexible nature of the
10 deoxyribose sugar. Furthermore, these data suggest that modifications resulting in higher conformationally biased sugar populations would have a greater influence on the structure of the surrounding deoxyribonucleotides and consequently a greater impact on human RNase H1 activity. The impact of the highly northern biased 2'-fluoro deoxyribonucleotide modification on the human RNase H1 activity of the surrounding
15 ribonucleotides shown here, suggests that conformational transmission exhibits a modest influence on human RNase H1 activity compared to other factors such as steric bulk in the minor groove and conformational flexibility within the phosphate backbone. It is important to note that the heteroduplexes examined here contained single nucleotide substitutions with a conformationally biased sugar. We have observed that substituting
20 contiguous stretches of modified nucleotides with conformationally biased sugars exhibits a greater influence on the human RNase H1 activity against adjacent deoxyribonucleotides (data not shown).

The structure of human RNase H1 shows that in addition to the catalytic domain shared with the *E. coli* homolog, human RNase H1 contains an RNA-binding domain at
25 the N-terminus of the protein (9). Human RNase H1 appears to identify the first 3'-DNA/5'-RNA base pair to achieve the proper positioning of the catalytic domain slightly less than one helical turn from the RNA-binding domain (20). Only when the enzyme is bound at the correct site and the helical geometry is appropriate will the catalytic unit be positioned appropriately to cleave the RNA. As a result, altering the local helical
30 geometry, (e.g., altering the minor groove width or the inter- and intranucleotide phosphate distances) at the catalytic site on the heteroduplex may have a global effect on the precise positioning of the catalytic region with respect to the RNA-binding domain of

human RNase H1 required for catalysis. Because the enzyme is predicted to position the catalytic domain 3' on the RNA relative to the RNA-binding domain, consistent with the results presented here, a local change in duplex geometry at the catalytic site on the substrate would impair the human RNase H1 activity at the adjacent 3'-ribonucleotides to the modification.

Implications for the design of antisense oligonucleotides

The demonstration that human RNase H1 plays a dominant role in the activities of DNA-like ASOs suggests that additional studies that explore the substrate preferences, enzymology, and regulatory processes for RNase H1 should support improved design of antisense agents. The demonstration that increases in RNase H1 activity correlated with increases in potency suggests that recruitment of RNase H1 to the ASO-RNA duplex and/or cleavage of the RNA by the enzyme is limiting for ASO activity. Any strategy that would improve these processes should improve ASO potency (Wu, in press).

We have shown that chimeric ASOs containing 2'-methoxyethoxy nucleotides in the wings and deoxyribonucleotides in the gap demonstrably enhance affinity for the target RNA and the nuclease stability. Despite the dramatic enhancement in binding affinity and nuclease stability, these chimeric ASOs only increase potency by 5 to 10-fold. This is due to a significant reduction in the catalytic efficiency of RNase H1 for these substrates. Thus strategies to enhance the interaction of human RNase H1 with the chimeric ASO-RNA complex are essential to increasing the potencies of this class of ASOs.

In that regard, the results presented here suggest that the preferred properties for the modified oligodeoxyribonucleotide include: 1) a conformationally flexible sugar producing an O_{4'}-endo pucker when hybridized to RNA; 2) no sterically bulky 2'-substituents; and 3) a conformationally rigid phosphate backbone. Clearly, the 2'-ara-fluoro, pseudouridine, 3'-methyl and Π -stacking modified deoxyribonucleotides exhibit many of these qualities. In light of the fact that none of the modifications tested were shown to enhance human RNase H1 activity compared with native deoxyribonucleotides and that these modification offer no clear advantage over native deoxyribonucleotides with respect to either duplex stability or nuclease resistance, other strategies to improve the potency ASO should be considered. For example, the calculated placement of these

modifications in chimeric ASOs may be an effective means to improve human RNase H1 activity by potentially blocking the conformational transmission of 2'-alkoxy deoxyribonucleotide into the deoxyribonucleotide region of the chimeric ASO.

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- 20 Table I shows the Relative initial cleavage rates and site-specific for the modified heteroduplex substrates. The position and description of the modifications are shown in Fig. 1. Initial and site-specific cleavage rates were determined as described in the Materials and Methods. ¹ Ratio site-specific cleavage rates represents the initial cleavage rates for the modified heteroduplexes divided by the unmodified substrate. ² Ratio V_0
- 25 represents the initial cleavage rates for the modified heteroduplexes divided by the unmodified substrate. ³ The designations for ribonucleotide positions are described in Figure 2. ⁴ Dashed lines indicate positions not cleaved by human RNase H1 for the unmodified substrate and modified heteroduplexes, i.e., terminal five 5'-ribonucleotides and five 3-ribonucleotides (see Fig. 2B). The V_0 values are an average of three
- 30 measurements with estimated errors of CV < 10%.

Example 11**Oligonucleotide and oligonucleoside synthesis**

The oligomeric compounds used in accordance with this invention may be conveniently and routinely made through the well-known technique of solid phase synthesis. Equipment for such synthesis is sold by several vendors including, for example, Applied Biosystems (Foster City, CA). Any other means for such synthesis known in the art may additionally or alternatively be employed. It is well known to use similar techniques to prepare oligonucleotides such as the phosphorothioates and alkylated derivatives.

10 Oligonucleotides: Unsubstituted and substituted phosphodiester (P=O) oligonucleotides are synthesized on an automated DNA synthesizer (Applied Biosystems model 394) using standard phosphoramidite chemistry with oxidation by iodine.

Phosphorothioates (P=S) are synthesized similar to phosphodiester oligonucleotides with the following exceptions: thiation was effected by utilizing a 10% w/v solution of 3,4-dihydro-2H-benzothio-1,1-dioxide in acetonitrile for the oxidation of the phosphite linkages. The thiation reaction step time was increased to 180 sec and preceded by the normal capping step. After cleavage from the CPG column and deblocking in concentrated ammonium hydroxide at 55°C (12-16 hr), the oligonucleotides were recovered by precipitating with >3 volumes of ethanol from a 1 M NH₄OAc solution.

20 Phosphinate oligonucleotides are prepared as described in U.S. Patent 5,508,270, herein incorporated by reference.

Alkyl phosphonate oligonucleotides are prepared as described in U.S. Patent 4,469,863, herein incorporated by reference.

3'-Deoxy-3'-methylene phosphonate oligonucleotides are prepared as described in U.S. Patents 5,610,289 or 5,625,050, herein incorporated by reference.

Phosphoramidite oligonucleotides are prepared as described in U.S. Patent, 5,256,775 or U.S. Patent 5,366,878, herein incorporated by reference.

Alkylphosphonothioate oligonucleotides are prepared as described in published PCT applications PCT/US94/00902 and PCT/US93/06976 (published as WO 94/17093 and WO 94/02499, respectively), herein incorporated by reference.

3'-Deoxy-3'-amino phosphoramidate oligonucleotides are prepared as described in U.S. Patent 5,476,925, herein incorporated by reference.

Phosphotriester oligonucleotides are prepared as described in U.S. Patent 5,023,243, herein incorporated by reference.

Borano phosphate oligonucleotides are prepared as described in U.S. Patents 5,130,302 and 5,177,198, both herein incorporated by reference.

5 Oligonucleosides: Methylenemethylimino linked oligonucleosides, also identified as MMI linked oligonucleosides, methylenedimethylhydrazo linked oligonucleosides, also identified as MDH linked oligonucleosides, and methylenecarbonylamino linked oligonucleosides, also identified as amide-3 linked oligonucleosides, and methyleneaminocarbonyl linked oligonucleosides, also identified as amide-4 linked oligo-
10 nucleosides, as well as mixed backbone oligomeric compounds having, for instance, alternating MMI and P=O or P=S linkages are prepared as described in U.S. Patents 5,378,825, 5,386,023, 5,489,677, 5,602,240 and 5,610,289, all of which are herein incorporated by reference.

Formacetal and thioformacetal linked oligonucleosides are prepared as described in
15 U.S. Patents 5,264,562 and 5,264,564, herein incorporated by reference.

Ethylene oxide linked oligonucleosides are prepared as described in U.S. Patent 5,223,618, herein incorporated by reference.

Example 12

20 RNA Synthesis

In general, RNA synthesis chemistry is based on the selective incorporation of various protecting groups at strategic intermediary reactions. Although one of ordinary skill in the art will understand the use of protecting groups in organic synthesis, a useful class of protecting groups includes silyl ethers. In particular bulky silyl ethers are used to
25 protect the 5'-hydroxyl in combination with an acid-labile orthoester protecting group on the 2'-hydroxyl. This set of protecting groups is then used with standard solid-phase synthesis technology. It is important to lastly remove the acid labile orthoester protecting group after all other synthetic steps. Moreover, the early use of the silyl protecting groups during synthesis ensures facile removal when desired, without undesired deprotection of
30 2' hydroxyl.

Following this procedure for the sequential protection of the 5'-hydroxyl in combination with protection of the 2'-hydroxyl by protecting groups that are differentially removed and are differentially chemically labile, RNA oligonucleotides were synthesized.

RNA oligonucleotides are synthesized in a stepwise fashion. Each nucleotide is added sequentially (3' - to 5'-direction) to a solid support-bound oligonucleotide. The first nucleoside at the 3'-end of the chain is covalently attached to a solid support. The nucleotide precursor, a ribonucleoside phosphoramidite, and activator are added, coupling the second base onto the 5'-end of the first nucleoside. The support is washed and any unreacted 5'-hydroxyl groups are capped with acetic anhydride to yield 5'-acetyl moieties. The linkage is then oxidized to the more stable and ultimately desired P(V) linkage. At the end of the nucleotide addition cycle, the 5'-silyl group is cleaved with fluoride. The cycle is repeated for each subsequent nucleotide.

Following synthesis, the methyl protecting groups on the phosphates are cleaved in 30 minutes utilizing 1 M disodium-2-carbamoyl-2-cyanoethylene-1,1-dithiolate trihydrate (S_2Na_2) in DMF. The deprotection solution is washed from the solid support-bound oligonucleotide using water. The support is then treated with 40% methylamine in water for 10 minutes at 55 °C. This releases the RNA oligonucleotides into solution, deprotects the exocyclic amines, and modifies the 2'-groups. The oligonucleotides can be analyzed by anion exchange HPLC at this stage.

The 2'-orthoester groups are the last protecting groups to be removed. The ethylene glycol monoacetate orthoester protecting group developed by Dharmacon Research, Inc. (Lafayette, CO), is one example of a useful orthoester protecting group which, has the following important properties. It is stable to the conditions of nucleoside phosphoramidite synthesis and oligonucleotide synthesis. However, after oligonucleotide synthesis the oligonucleotide is treated with methylamine which not only cleaves the oligonucleotide from the solid support but also removes the acetyl groups from the orthoesters. The resulting 2-ethyl-hydroxyl substituents on the orthoester are less electron withdrawing than the acetylated precursor. As a result, the modified orthoester becomes more labile to acid-catalyzed hydrolysis. Specifically, the rate of cleavage is approximately 10 times faster after the acetyl groups are removed. Therefore, this orthoester possesses sufficient stability in order to be compatible with oligonucleotide synthesis and yet, when subsequently modified, permits deprotection to be carried out

under relatively mild aqueous conditions compatible with the final RNA oligonucleotide product.

Additionally, methods of RNA synthesis are well known in the art (Scaringe, S. A. Ph.D. Thesis, University of Colorado, 1996; Scaringe, S. A., et al., *J. Am. Chem. Soc.*,
5 **1998**, *120*, 11820-11821; Matteucci, M. D. and Caruthers, M. H. *J. Am. Chem. Soc.*, **1981**,
103, 3185-3191; Beaucage, S. L. and Caruthers, M. H. *Tetrahedron Lett.*, **1981**, *22*, 1859-
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Tetrahedron Lett., **1994**, *25*, 4311-4314; Wincott, F. et al., *Nucleic Acids Res.*, **1995**, *23*,
2677-2684; Griffin, B. E., et al., *Tetrahedron*, **1967**, *23*, 2301-2313; Griffin, B. E., et al.,
10 *Tetrahedron*, **1967**, *23*, 2315-2331).

RNA antisense oligomeric compounds (RNA oligonucleotides) of the present invention can be synthesized by the methods herein or purchased from Dharmacon Research, Inc (Lafayette, CO). Once synthesized, complementary RNA antisense oligomeric compounds can then be annealed by methods known in the art to form double
15 stranded (duplexed) antisense oligomeric compounds. For example, duplexes can be formed by combining 30 μ l of each of the complementary strands of RNA oligonucleotides (50 μ M RNA oligonucleotide solution) and 15 μ l of 5X annealing buffer (100 mM potassium acetate, 30 mM HEPES-KOH pH 7.4, 2 mM magnesium acetate) followed by heating for 1 minute at 90°C, then 1 hour at 37°C. The resulting duplexed
20 antisense oligomeric compounds can be used in kits, assays, screens, or other methods to investigate the role of a target nucleic acid.

Example 13

Synthesis of Chimeric Oligonucleotides

25 Chimeric oligonucleotides, oligonucleosides or mixed oligonucleotides/oligonucleosides of the invention can be of several different types. These include a first type wherein the "gap" segment of linked nucleosides is positioned between 5' and 3' "wing" segments of linked nucleosides and a second "open end" type wherein the "gap" segment is located at either the 3' or the 5' terminus of the oligomeric compound.
30 Oligonucleotides of the first type are also known in the art as "gapmers" or gapped oligonucleotides. Oligonucleotides of the second type are also known in the art as "hemimers" or "wingmers".

**[2'-O-Me]--[2'-deoxy]--[2'-O-Me] Chimeric Phosphorothioate
Oligonucleotides**

Chimeric oligonucleotides having 2'-O-alkyl phosphorothioate and 2'-deoxy phosphorothioate oligonucleotide segments are synthesized using an Applied Biosystems automated DNA synthesizer Model 394, as above. Oligonucleotides are synthesized using the automated synthesizer and 2'-deoxy-5'-dimethoxytrityl-3'-O-phosphoramidite for the DNA portion and 5'-dimethoxytrityl-2'-O-methyl-3'-O-phosphoramidite for 5' and 3' wings. The standard synthesis cycle is modified by incorporating coupling steps with increased reaction times for the 5'-dimethoxytrityl-2'-O-methyl-3'-O-phosphoramidite. The fully protected oligonucleotide is cleaved from the support and deprotected in concentrated ammonia (NH₄OH) for 12-16 hr at 55°C. The deprotected oligo is then recovered by an appropriate method (precipitation, column chromatography, volume reduced *in vacuo* and analyzed spectrophotometrically for yield and for purity by capillary electrophoresis and by mass spectrometry.

**[2'-O-(2-Methoxyethyl)]--[2'-deoxy]--[2'-O-(Methoxyethyl)] Chimeric
Phosphorothioate Oligonucleotides**

[2'-O-(2-methoxyethyl)]--[2'-deoxy]--[2'-O-(methoxyethyl)] chimeric phosphorothioate oligonucleotides were prepared as per the procedure above for the 2'-O-methyl chimeric oligonucleotide, with the substitution of 2'-O-(methoxyethyl) amidites for the 2'-O-methyl amidites.

[2'-O-(2-Methoxyethyl)Phosphodiester]--[2'-deoxy Phosphorothioate]--[2'-O-(2-Methoxyethyl) Phosphodiester] Chimeric Oligonucleotides

[2'-O-(2-methoxyethyl phosphodiester)]--[2'-deoxy phosphorothioate]--[2'-O-(methoxyethyl) phosphodiester] chimeric oligonucleotides are prepared as per the above procedure for the 2'-O-methyl chimeric oligonucleotide with the substitution of 2'-O-(methoxyethyl) amidites for the 2'-O-methyl amidites, oxidation with iodine to generate the phosphodiester internucleotide linkages within the wing portions of the chimeric structures and sulfurization utilizing 3,4-dihydro-2H-benzothiole-3-one 1,1 dioxide (Beaucage Reagent) to generate the phosphorothioate internucleotide linkages for the center gap.

Other chimeric oligonucleotides, chimeric oligonucleosides and mixed chimeric oligonucleotides/oligonucleosides are synthesized according to United States patent 5,623,065, herein incorporated by reference.

It is intended that each of the patents, applications, and printed publications including books mentioned in this patent document be hereby incorporated by reference in their entirety.

5 As those skilled in the art will appreciate, numerous changes and modifications may be made to the preferred embodiments of the invention without departing from the spirit of the invention. It is intended that all such variations fall within the scope of the invention.

What is claimed is:

1. A compound of the formula:

5 $(T_2)_j-(T_3)_k-(T_1)_m-(T_4)_n-(T_1)_p-(T_5)_q-(T_2)_r$

wherein:

each T_1 is a 2'-deoxyribonucleotide;

each T_2 is a nucleotide having a higher binding affinity for a RNA target as
 10 compared to the binding affinity of a 2'-deoxyribonucleotide for said RNA target;
 each T_3 , T_4 and T_5 are transitional nucleotides;
 j and r independently are 0 to 10, and together the sum of j and r is at least 2;
 m and p independently are 1 to 20, and together the sum of m and p is at least 5;
 k , n and q independently are 0 to 3, and together the sum of k , n and q is at least 1.

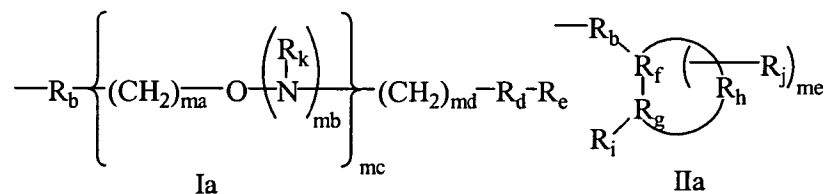
15

2. A compound of claim 1 wherein T_2 comprises a nucleotide having a northern conformation.

3. A compound of claim 2 wherein T_2 comprises a nucleotide having a 2'-
 20 modification.

4. A compound of claim 3 wherein said 2'-modification is hydroxyl, -O-alkyl, -O-alkyl-O-alkyl, S-alkyl, S-alkyl-O-alkyl, -F, -O-CH₂CH₂-O-CH₃, -O-CH₃, -O-CH₂-CH=CH₂ or a group having one of formula I_a or II_a:

25

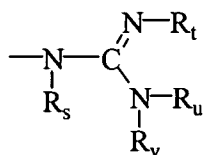


wherein:

R_b is O, S or NH;

R_d is a single bond, O, S or C(=O);

R_e is C_1 - C_{10} alkyl, $N(R_k)(R_m)$, $N(R_k)(R_n)$, $N=C(R_p)(R_q)$, $N=C(R_p)(R_r)$ or has formula IIIa;



IIIa

- 5 R_p and R_q are each independently hydrogen or C_1 - C_{10} alkyl;
 R_r is $-R_x-R_y$;
each R_s , R_t , R_u and R_v is, independently, hydrogen, $C(O)R_w$, substituted or unsubstituted C_1 - C_{10} alkyl, substituted or unsubstituted C_2 - C_{10} alkenyl, substituted or unsubstituted C_2 - C_{10} alkynyl, alkylsulfonyl, arylsulfonyl, a chemical functional group or a
10 conjugate group, wherein the substituent groups are selected from hydroxyl, amino, alkoxy, carboxy, benzyl, phenyl, nitro, thiol, thioalkoxy, halogen, alkyl, aryl, alkenyl and alkynyl;
or optionally, R_u and R_v , together form a phthalimido moiety with the nitrogen atom to which they are attached;
- 15 each R_w is, independently, substituted or unsubstituted C_1 - C_{10} alkyl, trifluoromethyl, cyanoethoxy, methoxy, ethoxy, t-butoxy, allyloxy, 9-fluorenylmethoxy, 2-(trimethylsilyl)-ethoxy, 2,2,2-trichloroethoxy, benzyloxy, butyryl, iso-butyryl, phenyl or aryl;
 R_k is hydrogen, a nitrogen protecting group or $-R_x-R_y$;
- 20 R_p is hydrogen, a nitrogen protecting group or $-R_x-R_y$;
 R_x is a bond or a linking moiety;
 R_y is a chemical functional group, a conjugate group or a solid support medium;
each R_m and R_n is, independently, H, a nitrogen protecting group, substituted or unsubstituted C_1 - C_{10} alkyl, substituted or unsubstituted C_2 - C_{10} alkenyl, substituted or
25 unsubstituted C_2 - C_{10} alkynyl, wherein the substituent groups are selected from hydroxyl, amino, alkoxy, carboxy, benzyl, phenyl, nitro, thiol, thioalkoxy, halogen, alkyl, aryl, alkenyl, alkynyl; NH_3^+ , $N(R_u)(R_v)$, guanidino and acyl where said acyl is an acid amide or an ester;

or R_m and R_n , together, are a nitrogen protecting group, are joined in a ring structure that optionally includes an additional heteroatom selected from N and O or are a chemical functional group;

R_i is OR_z , SR_z , or $N(R_z)_2$;

5 each R_z is, independently, H, C_1 - C_8 alkyl, C_1 - C_8 haloalkyl, $C(=NH)N(H)R_u$, $C(=O)N(H)R_u$ or $OC(=O)N(H)R_u$;

R_f , R_g and R_h comprise a ring system having from about 4 to about 7 carbon atoms or having from about 3 to about 6 carbon atoms and 1 or 2 heteroatoms wherein said heteroatoms are selected from oxygen, nitrogen and sulfur and wherein said ring system is
10 aliphatic, unsaturated aliphatic, aromatic, or saturated or unsaturated heterocyclic;

R_j is alkyl or haloalkyl having 1 to about 10 carbon atoms, alkenyl having 2 to about 10 carbon atoms, alkynyl having 2 to about 10 carbon atoms, aryl having 6 to about 14 carbon atoms, $N(R_k)(R_m)$ OR_k , halo, SR_k or CN;

m_a is 1 to about 10;

15 each m_b is, independently, 0 or 1;

m_c is 0 or an integer from 1 to 10;

m_d is an integer from 1 to 10;

m_e is from 0, 1 or 2; and

provided that when m_c is 0, m_d is greater than 1.

20

5. A compound of claim 1 where each of j and r are at least 2.

6. A compound of claim 3 wherein each of j and r are at least 2.

25 7. A compound of claim 1 wherein T_3 , T_4 and T_5 each comprise a nucleotide having one of an eastern or southern conformation.

8. A compound of claim 1 wherein T_3 , T_4 and T_5 each comprise a nucleotide having an eastern conformation.

30

9. A compound of claim 1 wherein at least one of T_3 , T_4 and T_5 comprise a 2'-fluoro-arabinonucleotide.

10. A compound of claim 3 wherein at least one of T_3 , T_4 and T_5 comprise a 2'-fluoro-arabinonucleotide.

5 11. A compound of claim 1 wherein each of T_3 , T_4 and T_5 comprise a 2'-fluoro-arabinonucleotide.

12. A compound of claim 1 wherein each of n and p are 0.

10 13. A compound of claim 10 wherein each of n and p are 0.

14. A compound of claim 11 wherein each of n and p are 0.

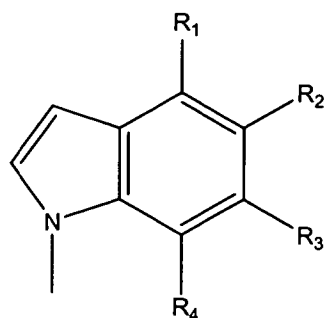
15 15. A compound of claim 1 wherein:
each T_2 comprises a nucleotide having a 2'-modification;
each of j and r are at least 2; and T_3 , T_4 and T_5 each comprise a nucleotide having one of an eastern or southern conformation.

20 16. A compound of claim 15 wherein T_3 , T_4 and T_5 each comprise a nucleotide having an eastern conformation.

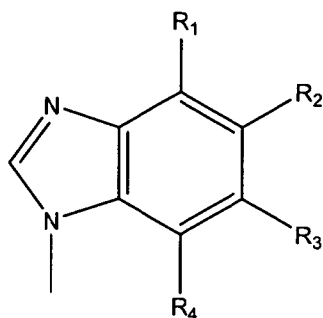
17. A compound of claim 1 wherein:
each of n and p are 0;
each T_2 comprises a nucleotide having a 2'-modification;
25 each of j and r are at least 2; and T_3 and T_5 each comprise a nucleotide having one of an eastern or southern conformation.

18. A compound of claim 17 wherein T_3 and T_5 each comprise a nucleotide having an eastern conformation.

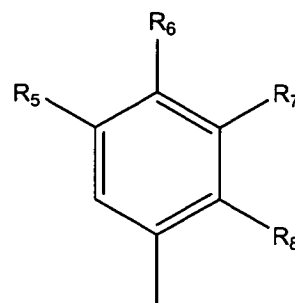
19. A compound of claim 16 wherein at least one of T₃, T₄ and T₅ comprise a 2'-fluoro-arabinonucleotide, an abasic nucleotide, a THF nucleoside, or a nucleotide having a nucleobase selected from Formulas I, II, and III:



I



II



III

5

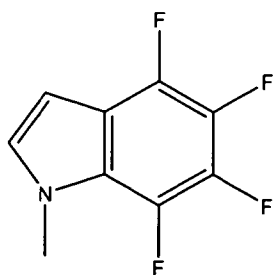
wherein:

each of R₁₋₈ is independently selected from H, halogen and C₁₋₃ alkyl.

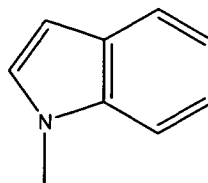
20. A compound of claim 19 wherein each of R₁₋₈ is independently selected from fluorine and methyl.

10

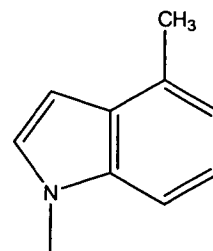
21. A compound of claim 19 wherein the nucleobase is selected from Formulas IV, V or VI:



IV



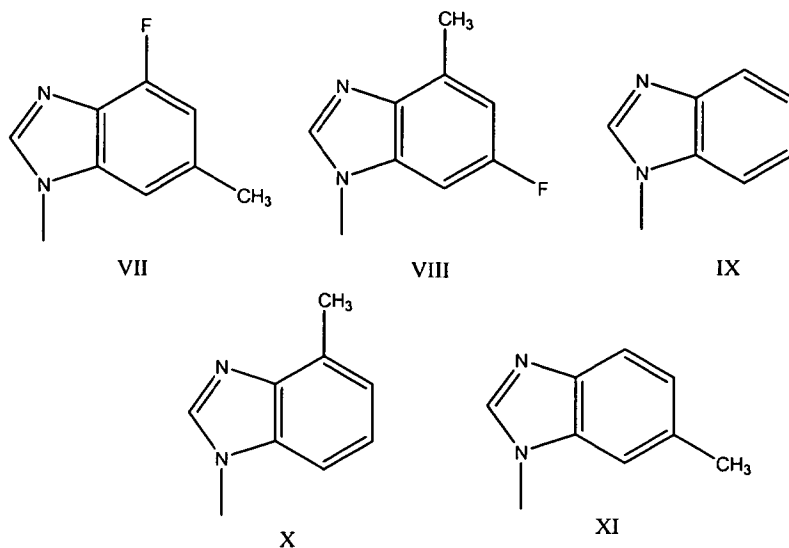
V



VI

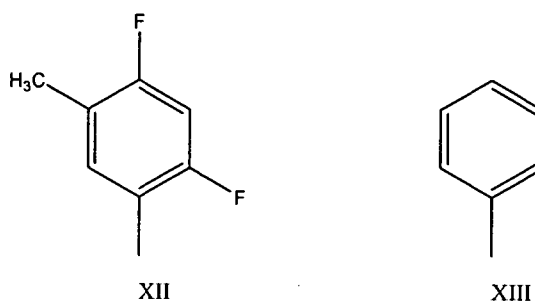
15

22. A compound of claim 19 wherein the nucleobase is selected from Formulas VII, VIII, IX, X or XI:



5

23. A compound of claim 19 wherein the nucleobase is selected from Formulas XII or XIII:



10

24. A compound of claim 15 or claim 17 wherein j and r are each from 2 to 5, and m is 10 to 16.

25. A compound of claim 15 or claim 17 wherein j is 2, r is 2 and m is 14-18.

26. A compound of claim 15 or claim 17 wherein j is 2, r is 2 and m is 16.

15 27. A compound of claim 15 or claim 17 wherein j is 4, r is 4 and m is 10-14.

28. A compound of claim 15 or claim 17 wherein j is 4, r is 4 and m is 12.

29. A compound of claim 15 or claim 17 wherein j is 5, r is 5 and m is 8-12.

30. A compound of claim 15 or claim 17 wherein j is 5, r is 5 and m is 10.

31. A method of increasing one of the rate of cleavage or the position of cleavage of a target RNA by RNase H comprising:

5 selecting an oligonucleotide having an RNase H cleaving region and a non-RNase H cleaving region;

selecting a transitional nucleotide capable of modulating transfer of the helical conformation characteristic of an oligonucleotide bound to its 3' hydroxy to an oligonucleotide bound to its 5' hydroxyl;

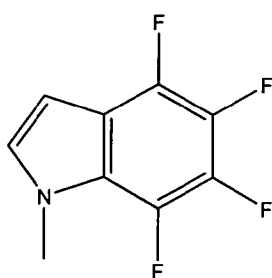
10 interspersing said transitional nucleotide in said oligonucleotide positioned between said RNase H cleaving region and said non-RNase H cleaving region; and

binding said oligonucleotide to said target RNA in the presence of RNase H.

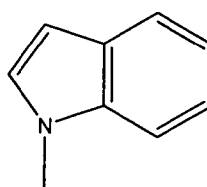
32. The method of claim 31 wherein the oligonucleotide has the formula of claim 1.

15

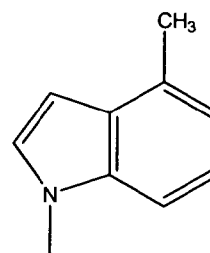
33. The method of claim 31 wherein the transitional nucleotide bears a nucleobase selected from Formulas IV, V or VI:



IV



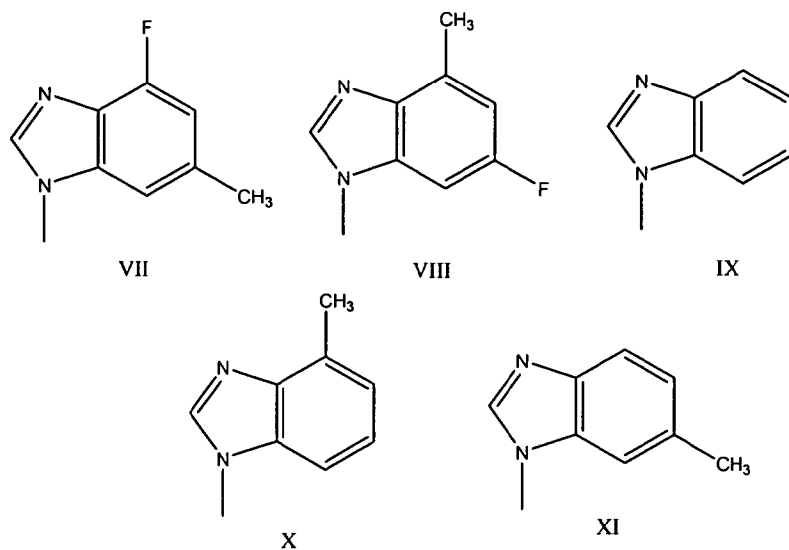
V



VI

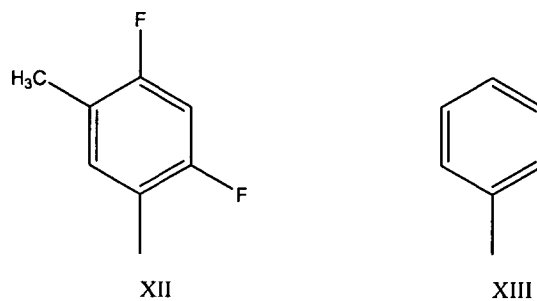
20

34. The method of claim 31 wherein the transitional nucleotide bears a nucleobase selected from Formulas VII, VIII, IX, X or XI:



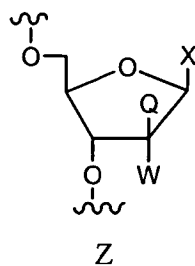
35. The method of claim 31 wherein the transitional nucleotide bears a nucleobase selected from Formulas XII or XIII:

5



36. The method of claim 31 wherein the transitional nucleotide has the Formula Z:

10



wherein:

X is selected from hydrogen, substituted or unsubstituted indolyl, substituted or unsubstituted benzimidazolyl, substituted or unsubstituted purinyl, or substituted or unsubstituted pyrimidinyl;

Q is selected from hydrogen or halogen;

5 W is selected from hydrogen or a 2'-substituent.

37. The method of claim 31 wherein the transitional nucleotide is a 2'-fluoro-arabinonucleotide.

10 38. An antisense oligonucleotide, comprising:
at least 2 conformationally different regions with a junction between each of said conformationally different regions, wherein each junction comprises at least one transitional nucleotide capable of modulating transfer of the helical conformation characteristic of an oligonucleotide bound to its 3'hydroxy to an oligonucleotide bound
15 to its 5' hydroxyl.

39. The antisense oligonucleotide of claim 38, wherein the conformationally different regions each comprise at least two nucleotides.

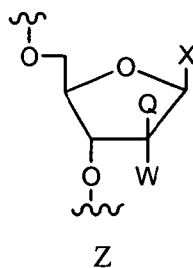
20 40. The antisense oligonucleotide of claim 39 wherein the nucleotides of each conformationally different region each possess the same type of sugar conformation.

41. The antisense oligonucleotide of claim 39, wherein the type of sugar conformation is selected from Eastern, Northern and Southern.

25

42. The antisense oligonucleotide of claim 38, wherein the transitional nucleotide is a base-modified nucleotide or a sugar-modified nucleotide.

43. The antisense oligonucleotide of claim 40, wherein the base-modified
30 nucleotide has the Formula Z:



wherein:

5 X is selected from hydrogen, substituted or unsubstituted indolyl, substituted or unsubstituted benzimidazolyl, substituted or unsubstituted purinyl, or substituted or unsubstituted pyrimidinyl;

Q is selected from hydrogen or halogen;

W is selected from hydrogen or a 2'-substituent.

10 44. The antisense oligonucleotide of claim 41, wherein the 2'-substituent is a substituted or unsubstituted aliphatic ether.

45. The antisense oligonucleotide of claim 43, wherein X is selected from Formulas I-XIII of claims 21-23.

15

46. The antisense oligonucleotide of claim 43, wherein W is H, and Q is F.

Abstract

The present invention provides compositions and methods for the optimization of cleavage of RNA species by RNase H. In some embodiments, the invention provides oligonucleotides that possess two or more regions of differing conformation, and at least
5 one transitional nucleobase positioned between the regions that is capable of modulating transfer of the helical conformation characteristic of the region bound to the 3'hydroxy thereof, to the region bound to the 5' hydroxyl thereof.

Fig. 1A

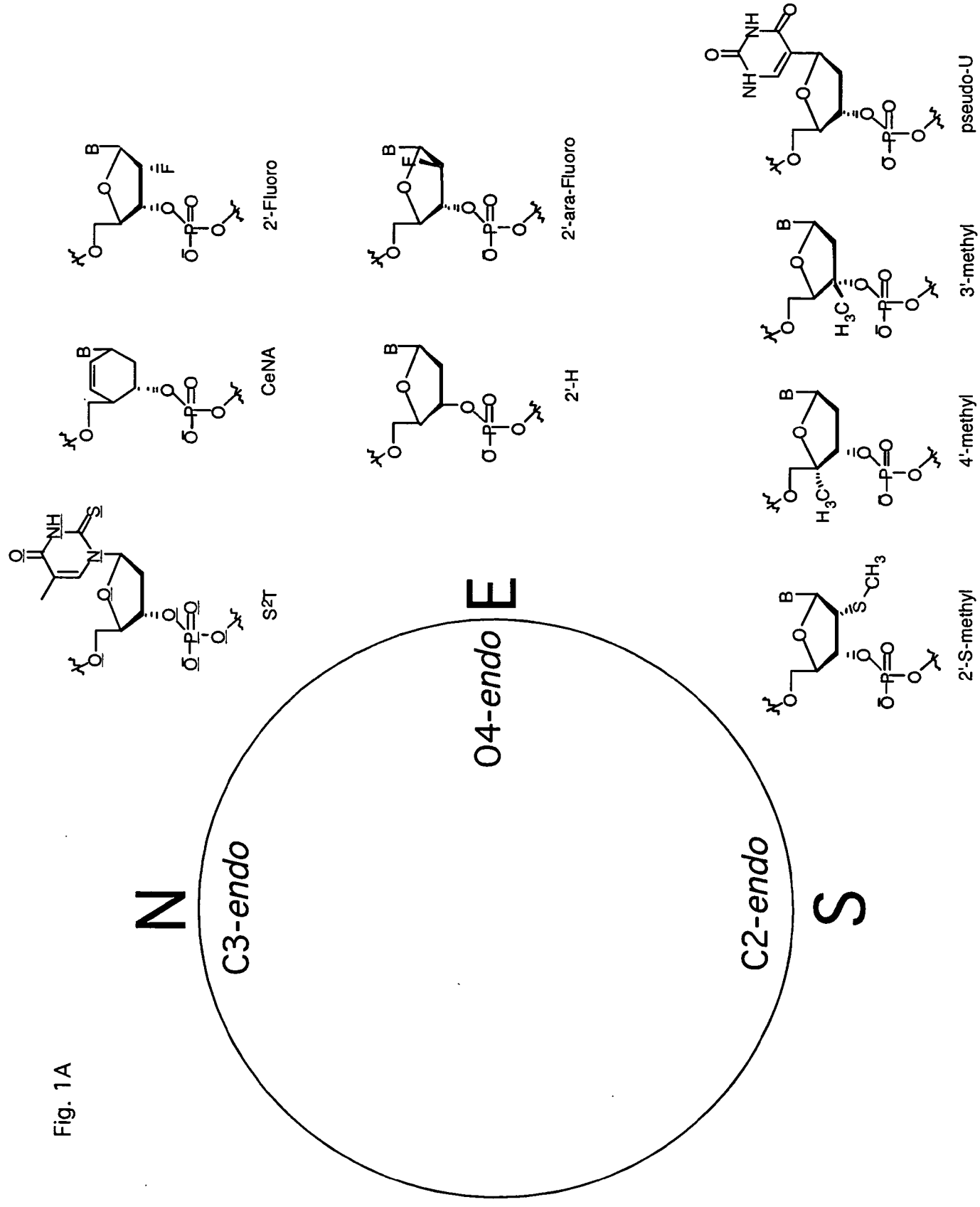


Fig. 1B

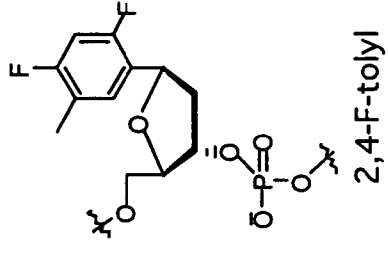
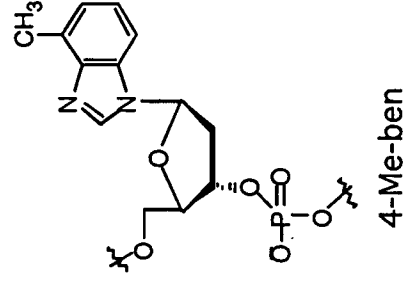
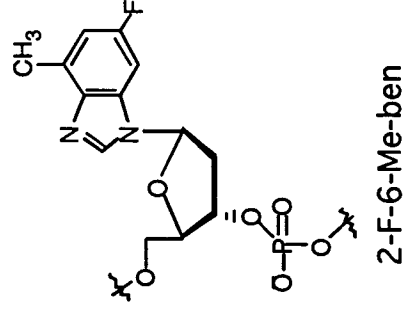
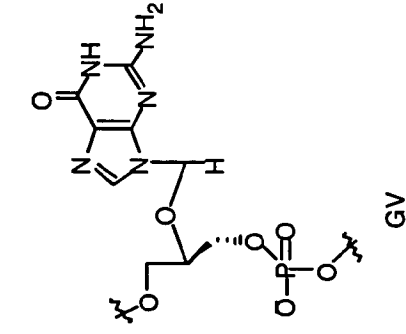
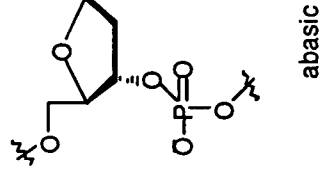
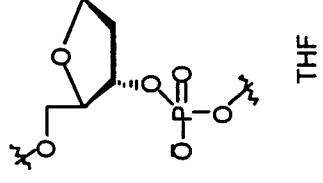
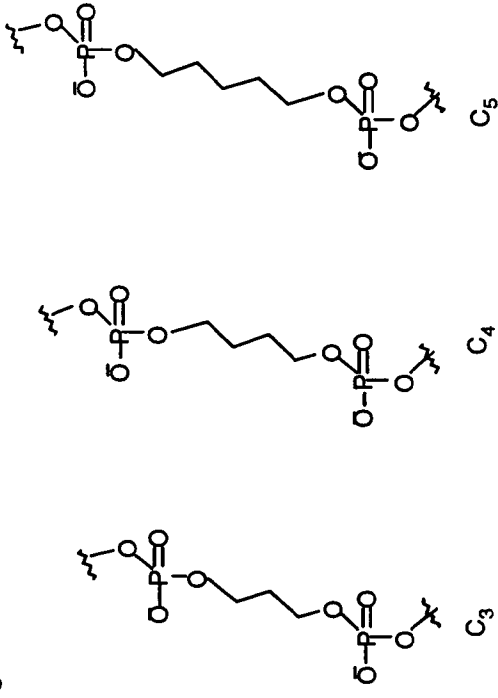


Fig. 1C

T_7 : CTACGCxTTCCACGCACAGT

T_8 : CTACGCTxTCCACGCACAGT

T_9 : CTACGCTTxCCACGCACAGT

C_{10} : CTACGCTTTxCACGCACAGT

C_{11} : CTACGCTTTCxACGCACAGT

A_{12} : CTACGCTTTCCxCGCACAGT

C_{13} : CTACGCTTTCCAxGCACAGT

G_{14} : CTACGCTTTCCACxCACAGT

C_{15} : CTACGCTTTCCACGxACAGT

Fig. 2A

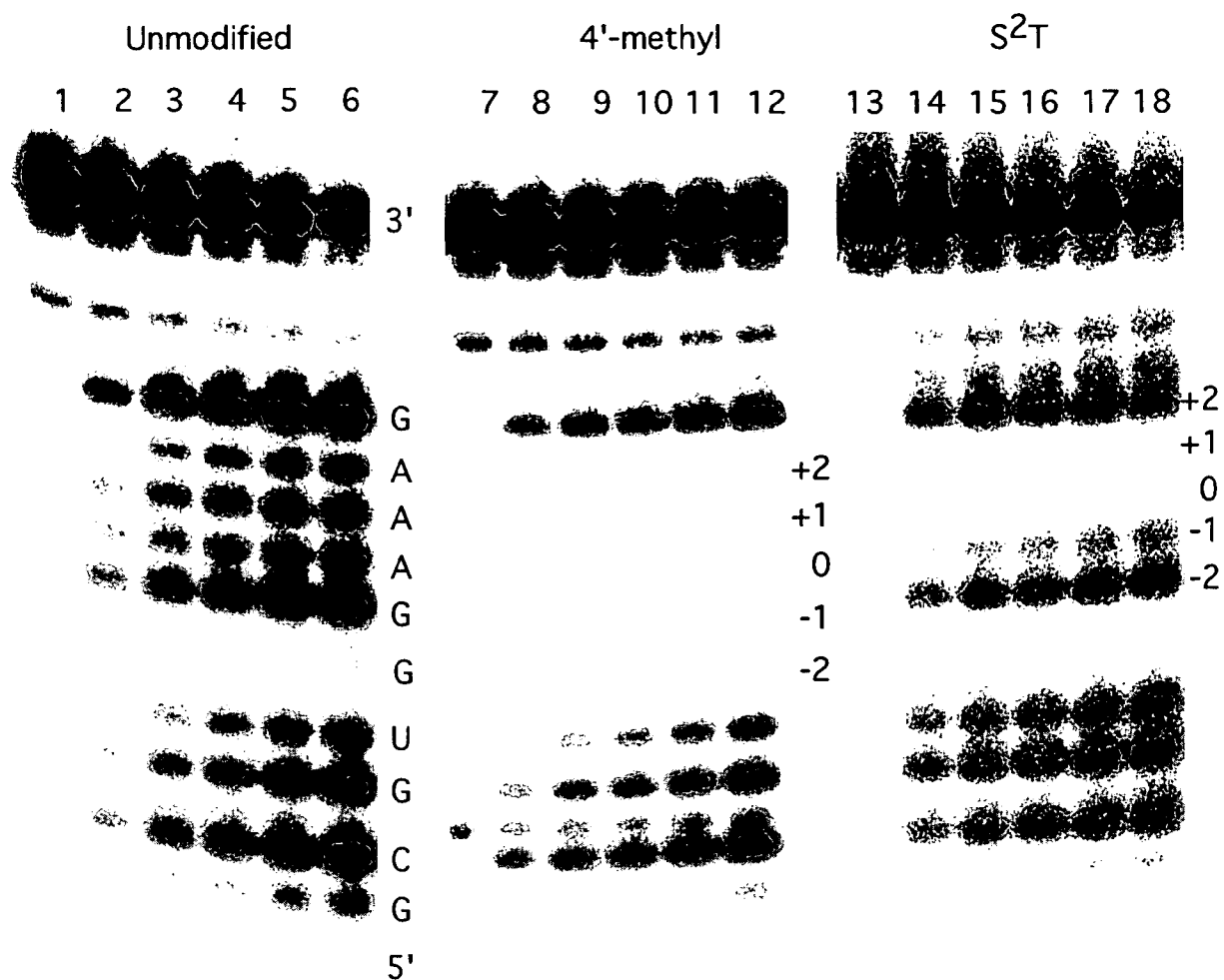


Figure 2B

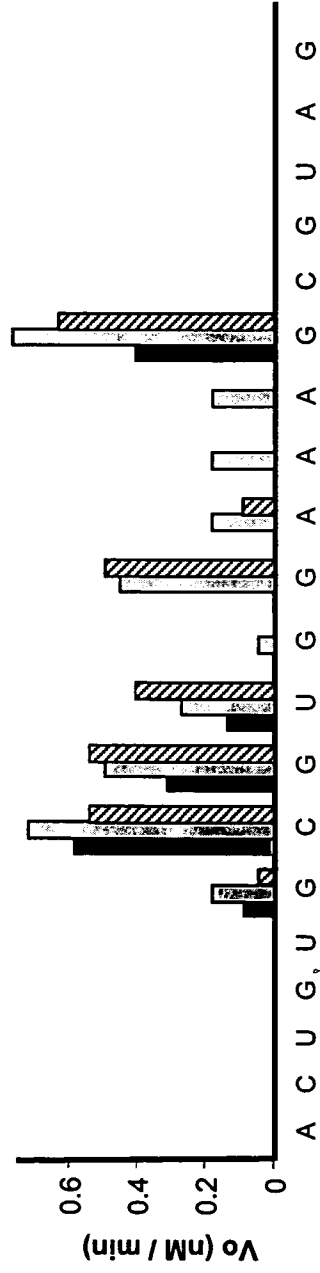


Fig. 3

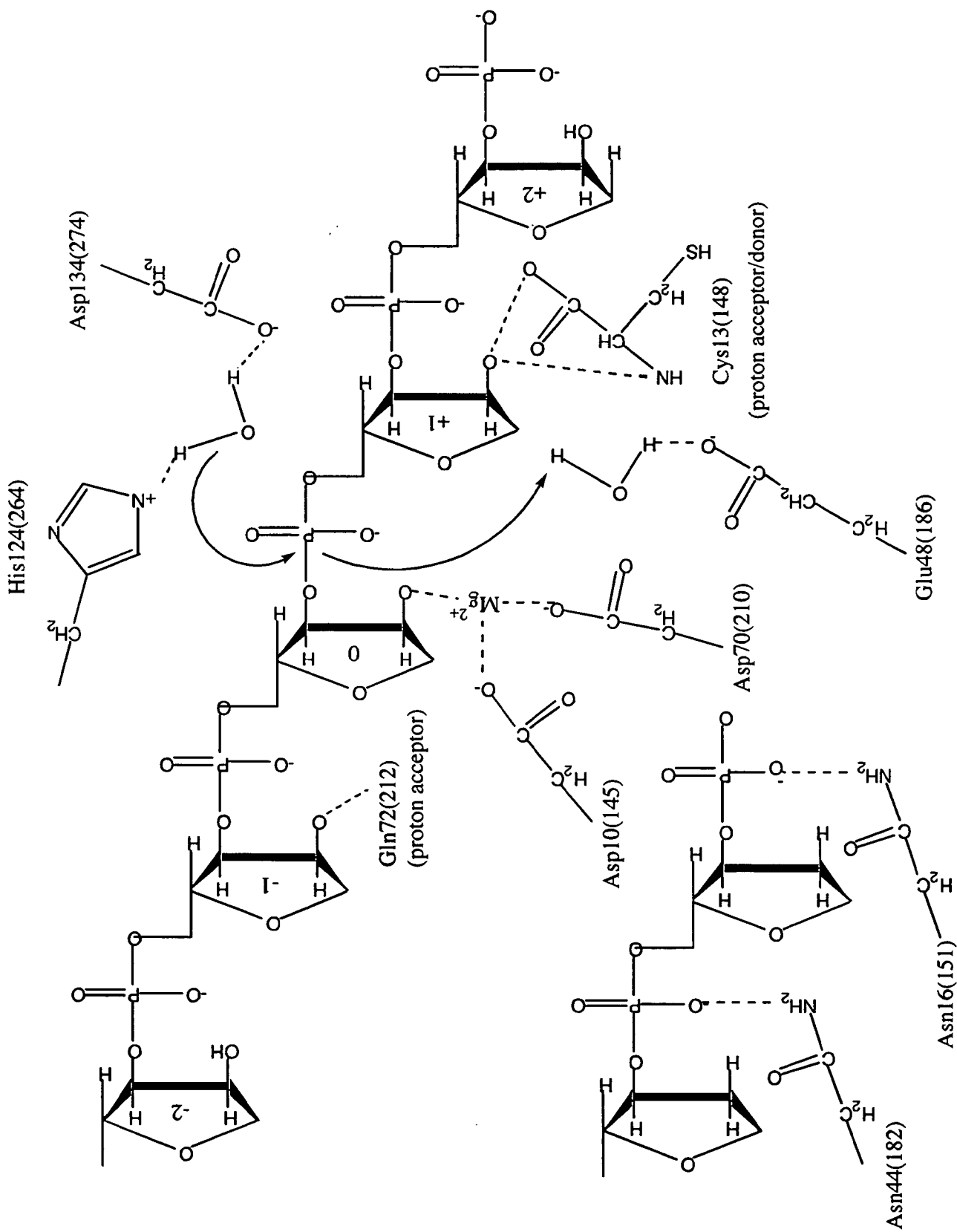


Figure 4a

Table IA

| Modification | Position of modification | Ratio Site-Specific Cleavage Rate ¹ (modified/ unmodified) | | | | | Ratio V_0^2 (modified/unmodified) |
|------------------|--------------------------|---|-----------------|----------------|-----|-----------------|-------------------------------------|
| | | -2 | -1 | 0 ³ | +1 | +2 | |
| 2'-ara-Fluoro | T ₇ | 0.9 | 1.1 | 0.9 | 1.0 | -- ⁴ | 1.1 |
| | T ₈ | 1.3 | 1.5 | 0.8 | 1.5 | 1.50 | 1.1 |
| | T ₉ | 1.0 | 1.1 | 1.5 | 0.6 | 0.4 | 1.1 |
| | C ₁₀ | 0.7 | 1.1 | 0.8 | 1.0 | 0.6 | 1.2 |
| | C ₁₃ | 1.1 | 0.7 | 0.6 | 0.8 | 0.6 | 1.1 |
| | C ₁₅ | -- ⁴ | -- ⁴ | 1.1 | 1.3 | 0.4 | 1.1 |
| Pseudouridine | T ₇ | 0.8 | 0.7 | 0.6 | 0.6 | -- ⁴ | 0.9 |
| | T ₈ | 1.4 | 1.3 | 0.7 | 0.2 | 0.3 | 1.1 |
| | T ₉ | 1.0 | 0.8 | 1.0 | 0.2 | 0.2 | 1.2 |
| 2'-Fluoro | T ₇ | 0.5 | 0.2 | 0.2 | 0.1 | -- ⁴ | 0.6 |
| | T ₈ | 0.8 | 0.6 | 0.2 | 0.3 | 0.6 | 0.7 |
| | T ₉ | 1.1 | 0.9 | 0.2 | 0.3 | 0.6 | 0.7 |
| S ² T | T ₇ | 0.4 | 0.4 | 0.2 | 0.1 | -- ⁴ | 0.5 |
| | T ₈ | 1.1 | 0.6 | 0.0 | 0.0 | 0.7 | 0.7 |
| | T ₉ | 1.3 | 1.0 | 0.3 | 0.3 | 0.9 | 0.7 |
| 2'-S-Methyl | T ₇ | 0.6 | 0.6 | 0.1 | 0 | -- ⁴ | 0.5 |
| | T ₈ | 0.5 | 0.6 | 0.1 | 0 | 0 | 0.4 |
| | T ₉ | 0.7 | 0.6 | 0.2 | 0.1 | 0.3 | 0.4 |
| 4'-Methyl | T ₇ | 0.5 | 0.3 | 0 | 0 | -- ⁴ | 0.5 |
| | T ₈ | 0.3 | 0.1 | 0 | 0 | 0 | 0.6 |
| | T ₉ | 0 | 0 | 0 | 0 | 0 | 0.3 |
| 3'-Methyl | T ₇ | 0.3 | 0.7 | 1.0 | 0.8 | -- ⁴ | 0.8 |
| | T ₈ | 1.6 | 0.8 | 0.7 | 0.1 | 0.1 | 0.8 |
| | T ₉ | 1.3 | 0.7 | 0.6 | 0.2 | 0 | 0.7 |
| CeNa | T ₇ | 0.6 | 0.8 | 0.8 | 0 | -- ⁴ | 0.4 |
| | T ₈ | 0.7 | 0.9 | 0.6 | 0 | 0 | 0.4 |
| | T ₉ | 0.3 | 1.0 | 0.5 | 0 | 0 | 0.4 |

Figure 4B

Table IB

| Modification | Position of modification | Ratio Site-Specific Cleavage Rate ¹ (modified/ unmodified) | | | | | Ratio V ₀ ² (modified/unmodified) |
|-----------------|--------------------------|---|-----------------|----------------|-----|-----|---|
| | | -2 | -1 | 0 ³ | +1 | +2 | |
| Propyl linker | A ₁₂ | 0.6 | 0 | 0 | 0 | 0 | 0.5 |
| | G ₁₄ | -- ⁴ | 0 | 0 | 0 | 0 | 0.6 |
| | C ₁₅ | -- ⁴ | -- ⁴ | 0 | 0 | 0 | 0.5 |
| Butyl linker | A ₁₂ | 0.5 | 0.1 | 0 | 0 | 0 | 0.5 |
| | C ₁₅ | -- ⁴ | -- ⁴ | 0.1 | 0 | 0.6 | 0.5 |
| Pentyl linker | A ₁₂ | 0.1 | 0.1 | 0 | 0.5 | 0.9 | 0.6 |
| | C ₁₅ | -- ⁴ | -- ⁴ | 0.1 | 0 | 0.9 | 0.5 |
| Tetrahydrofuran | A ₁₂ | 0 | 0 | 0 | 0.6 | 0.5 | 0.4 |
| | G ₁₄ | -- ⁴ | 0.5 | 0 | 0.1 | 0 | 0.4 |
| Abasic | T ₉ | 0.6 | 0.3 | 0.2 | 0.3 | 0.2 | 0.6 |
| | C ₁₀ | 0.5 | 0.4 | 0.5 | 0.3 | 0.1 | 0.6 |
| | C ₁₁ | 0.6 | 0.3 | 0.2 | 0.3 | 0.2 | 0.5 |
| Gancyclovir | G ₁₄ | -- ⁴ | 0.1 | 0.5 | 0.3 | 0.4 | 0.4 |
| 4-F-6-Me-ben | G ₁₄ | -- ⁴ | 0.8 | 0.8 | 0.9 | 0.9 | 0.8 |
| 4-Me-ben | A ₁₂ | 0.7 | 0.7 | 0.9 | 0.9 | 0.5 | 0.8 |
| 2,4-F-tolyl | T ₉ | 0.9 | 1.0 | 1.3 | 0.8 | 0.2 | 0.8 |
| | C ₁₀ | 1.3 | 1.5 | 1.0 | 1.0 | 0.1 | 0.9 |
| | C ₁₁ | 1.3 | 1.1 | 0.8 | 0.7 | 0.5 | 1.1 |